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# Genetic analysis of Limb Girdle Muscular Dystrophy and Miyoshi Myopathy

Gillian Summerill

MSc 2003

## Abstract

The autosomal recessive muscular dystrophies encompass limb girdle muscular dystrophy (LGMD) and Miyoshi myopathy (MM), which can show clinical and genetic overlap. In this study we have made good progress towards understanding the molecular basis of LGMD2I and non-dysferlin MM.

Our work on LGMD2I involved haplotype analysis of chromosome 19q13.3 to identify potential LGMD2I families. We generated a primary transcript map of the LGMD2I region. During this work, mutations in FKRP were described in LGMD2I and MDC1C. In 3 of our families showing potential linkage to 19q13.3, FKRP mutations were identified. A common mutation in FKRP, C826A (Leu276Ile), is associated with LGMD2I and this mutation was present in 2 of our families and in >70% of the LGMD2I families studied. Genotype-phenotype correlations show this mutation is associated with a milder disease course. FKRP is a predicted glycosyltransferase and mutations in its gene appear to be a common cause of muscular dystrophy.

Our work on the analysis of non-dysferlin MM supports the existence of a MM gene, designated MMD2, on chromosome 10p. We have defined a 25Mb region on chromosome 10 shared by affecteds in 2 non-dysferlin MM families. In 5 further families, additional haplotype analysis is required to confirm exclusion from chromosome 10p.

In the non-dysferlin MM families haplotype analysis of the caveolin 3 region on chromosome 3 has suggested no involvement of caveolin 3 in non-dysferlin MM. SSCP analysis and sequencing has failed to identify mutation in caveolin 3.

The confirmation of the MMD2 gene and identification of recombinant boundaries, allowing candidate gene analysis, will allow us to identify the MMD2 gene.

**Genetic Analysis of Limb Girdle Muscular Dystrophy  
and Miyoshi Myopathy**

**by**

**Gillian Summerill**

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**A thesis submitted to the School of Biological and Biomedical Sciences**

**University of Durham**

**In accordance with the requirements for the degree of**

**Master of Science**

**December 2003**



**14 OCT 2004**

**For John and Ben**

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## **Declaration**

I declare that the work contained within this thesis submitted by me for the degree of Master of Science is my own original work, except where otherwise stated, and has not been submitted previously for a degree at this or any other University.

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## **CHAPTER 1**

### **INTRODUCTION**

## **1.1 THE MUSCULAR DYSTROPHIES**

The muscular dystrophies are a group of clinically and genetically heterogeneous disorders, characterised by a progressive weakening and wasting of muscles. Since the 1950's more than 20 types of muscular dystrophy have been categorised. These include:

- Duchenne/Becker muscular dystrophy (DMD/BMD)
- Emery-Dreifuss muscular dystrophy (EDMD)
- Congenital muscular dystrophy (CMD)
- Limb-girdle muscular dystrophy (LGMD)
- Facioscapulohumeral muscular dystrophy (FSHD)
- Myotonic dystrophy (DM)

There are also many related neuromuscular conditions involving muscles and motor nerves. All of the above muscular dystrophies are caused by gene defects which may be inherited in an autosomal dominant, autosomal recessive or X-linked manner. Muscular dystrophy can also arise by spontaneous mutation. (Emery, AEH., ed. Neuromuscular disorders: Clinical and Molecular Genetics (Wiley, 1998) pp. 1-18).

## **1.2 DYSTROPHIN & DMD/BMD**

The most common of all the muscular dystrophies affecting children in the UK are Duchenne and Becker muscular dystrophies. Molecular genetic evidence showed that both disorders segregate with the same Xp21 chromosomal region and that DMD and BMD were most likely allelic disorders caused by mutation in the same gene (Kingston et

al, 1983). Genomic deletions were later detected in region Xp21 for both DMD and BMD patients (Kunkel et al, 1985). As inheritance of the disorder is X-linked, patients are therefore typically male only.

The dystrophin gene, which is located at chromosome Xp21, was identified by a positional cloning approach (Murray et al., 1982). It is one of the largest genes known, with at least 79 exons coding for a cDNA of 14.6 kb which spans over 2.5 Mb of DNA. The gene is arranged into four domains: domain A, exons 1-7, actin binding; domain B, exons 8-44, rod domain; domain C, exons 45-55, cysteine rich; domain D, exons 56-79, C-terminal (Koenig et al., 1988).

The protein product dystrophin was identified by immunobiochemical and immunohistochemical studies (Hoffman et al., 1987), and has been shown to be a 427 kDa rod shaped spectrin like protein (Koenig et al., 1988). Dystrophin has been localised to the sarcolemma (Zubrzycka-Gaarn et al., 1988), existing as a complex associated with the dystroglycans, syntrophins and sarcoglycans (Campbell and Kahl, 1989; Ervasti and Campbell, 1991). The protein was found to be absent in DMD patients, and reduced and/or altered in BMD patients (Hoffman et al., 1987, 1988; Arahata et al., 1989). Fig. 1.1 shows the dystrophin-glycoprotein complex and its arrangement at the muscle cell membrane.

The most common dystrophin mutations identified in DMD/BMD are deletions, followed by duplications. The resulting frameshift results in a premature termination, causing the loss of most of the dystrophin from the muscle cell membrane, which is thought to be anchored via its C-terminal D-domain (Koenig et al., 1988; Ervasti and Campbell, 1991; Matsumura and Campbell 1993, 1994). In over 90% of cases this genotype results in the

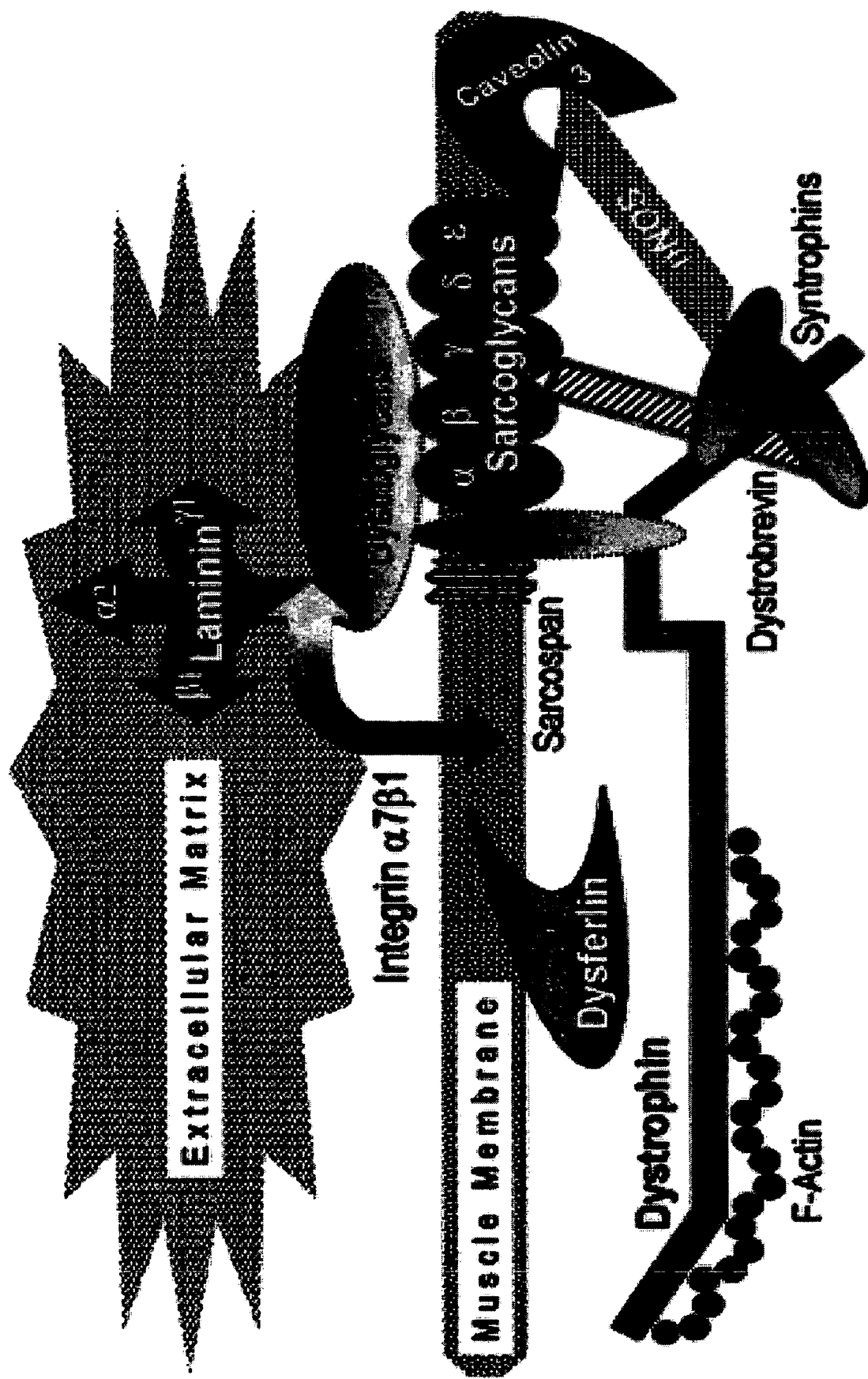


Fig. 1.1 The dystrophin-glycoprotein complex

Duchenne phenotype. This runs a much more severe clinical course than the Becker phenotype, although the two are clinically indistinguishable. Mutations which are in frame but generate a larger or smaller dystrophin molecule do not result in complete loss of the protein from the membrane. 10-40% of the protein is still produced, and this results in the milder Becker phenotype. Cases of very short dystrophins have been described, leading to mild BMD (Passos-Bueno et al., 1994). These patients usually have deletions of exon 45 to 48. 30% of the deletions seen are in exons 2-20, and 70% are in exons 44-53.

Muscle biopsies show a marked variation in muscle fibre size and shape, and alterations in fibre type proportions. DMD/BMD patients tend to have a predominance of type I or slow twitch fibres and there is a deficiency of type II or fast twitch fibres. Becker patients have both type I and type II fibres. Deposition of connective and adipose tissue is seen, along with muscle degeneration and necrosis.

Onset of DMD and BMD is usually between 3 and 6 years of age. Patients present with difficulty in walking or running, may be unable to hop or jump and have difficulty in climbing stairs. They may also show difficulty in rising from the floor, and find it necessary to use their arms to push themselves up. This characteristic method of standing is known as the Gowers sign, and is routinely used as a clinical tool in initially diagnosing DMD and BMD. These difficulties are caused by the symmetrical weakness of the hip and proximal limb muscles which is seen at onset of the disease. Calf hypertrophy may also be a feature, and with progression of the disease muscle weakness spreads to the upper limbs and neck. The cardiac and respiratory muscles also become involved (Nigro et al., 1990), and mental retardation is seen in a third of Duchenne



patients (Bresolin et al., 1994). Loss of ambulation is around the age of 13 years for Duchenne boys, and 16 years for Becker. Both disorders are fatal, with death usually occurring in the early to mid twenties due to cardiac arrest or respiratory failure.

### **1.3 THE LIMB-GIRDLE MUSCULAR DYSTROPHIES**

The limb-girdle muscular dystrophies are a group of clinically and genetically heterogeneous progressive muscle disorders. At one time a diagnosis of LGMD would be based mainly on exclusion from other forms of muscular dystrophy such as the X-linked diseases, FSHD or CMD. LGMD at onset can be characterised by weakness of the proximal shoulder or pelvic girdle musculature, and may occur at any age. Early contractures may be a feature and calf hypertrophy is sometimes evident at onset. Progression of the disease varies from mild to severe and muscle involvement is variable. LGMD is therefore phenotypically similar to many other forms of muscular dystrophy or neuromuscular disorders, such as BMD, manifesting carriers of X-linked muscular dystrophies, spinal muscular atrophy and mitochondrial and metabolic myopathies.

Research into identification of the causative genes for this group of muscular dystrophies has led to a greater understanding of the heterogeneity observed within the group and has facilitated the possibility of precise molecular diagnoses in many cases.

To date, 13 types of limb-girdle muscular dystrophy have been characterised, three inherited as autosomal dominant and ten as autosomal recessive. These have been mapped to many different chromosomes, and in most cases the gene product has been identified. Table 1.1 summarises this information, which has served to highlight the

INHERITANCE	LOCUS NAME	GENE LOCUS	GENE PRODUCT	ALTERNATIVE NAME	MUTATIONS, TYPE AND DISTRIBUTION
Autosomal dominant	LGMD1A	5q22-24	Myotilin		
	LGMD1B	1q11-21	Lamin A/C		
	LGMD1C	3p25	Caveolin 3 (CAV3)		
	LGMD1D	7q36.2-36.3			
Autosomal recessive	LGMD2A	15q15.1-21.2	Calpain 3 (CAPN3)	Calpainopathy	M, small De and SS mutations, described throughout gene. Founder mutations in Amish, Reunion, Basque and Turkish pop. Occasional recurrent mutations.
	LGMD2B	2p13-16	Dysferlin (DYSF)	Dysferlinopathy, Miyoshi Myopathy	M, Du, small De, appear to be across gene. Apparent founder mutation in Libyan Jews.
	LGMD2G	17q11-12	Telethonin		
	LGMD2H	9q31-33	TRIM32?		Transition, missense. D487N.
	LGMD2I	19q13.3	FKRP		C826A common mutation.
	LGMD2D	17q12-21.33	$\alpha$ -Sarcoglycan (SGCA)	$\alpha$ -Sarcoglycanopathy	N, M, Du, small SS mutations. Exon 3 common site. Several recurrent mutations. R77C frequent (~40% of mutations).
	LGMD2E	4q12	$\beta$ -Sarcoglycan (SGCB)	$\beta$ -Sarcoglycanopathy	M and truncating mutations. M clustered in extracellular domain. Founder mutation in Amish population (T151R).
	LGMD2C	13q13	$\gamma$ -Sarcoglycan (SGCG)	$\gamma$ -Sarcoglycanopathy	Small De, few M. Founder mutations in North African (del 521T) and European gypsy population (C283Y).
	LGMD2F	5q33-34	$\delta$ -Sarcoglycan (SGCD)	$\delta$ -Sarcoglycanopathy	Single nucleotide substitution or deletions.

Key to abbreviation of mutations: Nonsense = N, Missense = M, Duplication = Du, Deletion = De, Splice site = SS

Table 1.1 Types of Limb-Girdle Muscular Dystrophy defined by their genetic basis with causative mutations where known.

different mechanisms by which muscular dystrophy can arise and has given some insight into the observed clinical heterogeneity.

In light of this new information the LGMD phenotypes were reclassified by a consortium meeting under the auspices of the European Neuromuscular Centre and a locus-based classification was adopted (Bushby and Beckmann, 1995). This meant that conditions such as ‘severe childhood autosomal recessive muscular dystrophy’ (SCARMD), of which adult onset cases have been recognised, could be incorporated more accurately into the new classification by the loci identified for the group.

The limb-girdle muscular dystrophies are divided into two groups according to their mode of inheritance – autosomal dominant or autosomal recessive. The autosomal dominant group was termed LGMD1, the three forms contained within it mapping to three different loci. These loci were designated A, B and C. Similarly, the autosomal recessive group was termed LGMD2, with the various loci designated LGMD2A, LGMD2B etc. in the order of their identification (Bushby and Beckmann, 1995).

### 1.3.1 THE AUTOSOMAL DOMINANT LGMDs

Only a small proportion of limb girdle muscular dystrophies have dominant inheritance, and it is believed that at least some of these cases are misdiagnosed and are actually cases of the dominantly inherited muscular dystrophy facioscapulohumeral MD (van der Kooi *et al.*, 1994), which can present with prominent pelvic girdle weakness and limited facial muscle involvement. However it is now possible to diagnose most cases of FSHD by

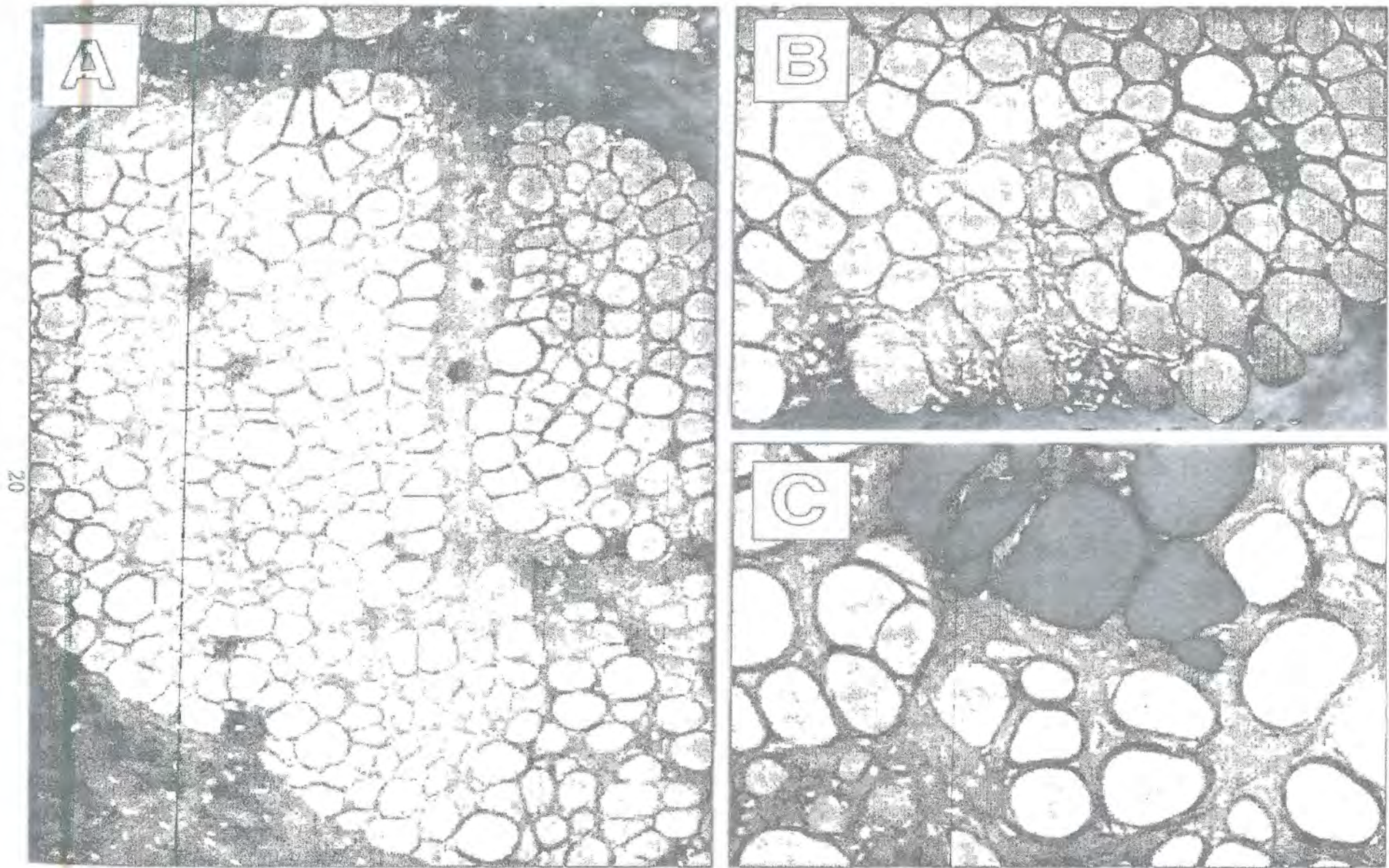
DNA analysis, and the three forms of autosomal dominant limb girdle muscular dystrophies have been genetically determined.

An important distinguishing feature between the autosomal dominant LGMD patients and the autosomal recessive is the much lower serum creatine kinase levels seen with the dominantly inherited forms.

### **LGMD1A**

The gene for LGMD1A maps to chromosome 5q22-24, and was identified following a study of a large North American pedigree (Gilchrist *et al.*, 1988; Speer *et al.*, 1992). The gene product has been identified as myotilin, mutations in which were observed in a large North American family of German descent (Hauser *et al.*, 2000), and an Argentinian pedigree (Hauser *et al.*, 2002). Myotilin is a sarcomeric protein that binds to alpha-actinin, filamin c and F-actin, and is localised in the Z-line. The disease association and functional characteristics of myotilin suggest a role in stabilisation and anchorage of thin filaments, which may be a prerequisite for correct Z-disc organisation. Histologically, muscle of affected individuals shows degeneration of myofibres, variations in fibre size, fibre splitting, centrally located myonuclei and a large number of autophagic vesicles (Fig. 1.2). Affected muscle also shows disorganisation and streaming of the Z-line similar to that seen in nemaline myopathy.

LGMD1A patients present with a predominantly proximal muscular dystrophy, with onset ranging from 18 to 35 years, with some suggestion of anticipation (Speer *et al.*, 1994). Progression of the disease is slow with few being confined to a wheelchair.



**Fig. 1.2 Progressive dystrophic changes of affected skeletal muscle**  
 Histologically, muscle of affected individuals shows degeneration of myofibres, variations in fibre size (A), centrally located myonuclei (B), fibre splitting (C), and a large number of autophagic vesicles.

Symptoms include dysarthria and Achilles tendon contractures, some arm weakness but only in association with leg weakness, and distal weakness sometimes becoming a feature with progression of the disease. Creatine kinase levels are only mildly elevated.

## **LGMD1B**

Mapping to chromosome 1q11-21 (van der Kooi *et al.*, 1997), this form of muscular dystrophy presents between 4 and 38 years with proximal lower limb weakness and variable calf hypertrophy. The disease is slowly progressive, with Achilles tendon or elbow contractures either minimal or becoming a feature late in the course of the disease. Creatine kinase levels are normal or mildly elevated. LGMD1B is also associated with cardiac involvement (van der Kooi *et al.*, 1996), and sudden death is common if the condition is untreated. Before the age of 25 most patients have normal atrioventricular conduction, but around a third develop first degree heart block between 25 and 35 years. This progresses to second degree heart block between 35 and 45 years of age, often necessitating the insertion of a pacemaker. Dilated cardiomyopathy may also become a feature. This phenotype is similar to one described by Fang and colleagues in 1997 (Fang *et al.*, 1997).

The autosomal dominant Emery-Dreifuss muscular dystrophy also links to the same region of chromosome 1q, the corresponding gene being LMNA which encodes lamins A/C, two proteins of the nuclear envelope that interact directly with emerin. Mutations in the LMNA gene have been found to cause at least four different kinds of genetic disorders: dilated cardiomyopathy type 1A (CMD1A) and familial partial lipodystrophy



(FPLD), as well as autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) and limb-girdle muscular dystrophy type 1B (LGMD1B).

Sufferers of EDMD show a similar cardiac involvement to those of LGMD1B, although the pattern of muscle involvement is dissimilar (Emery, 1989; Rudenskaya *et al.*, 1994). This phenotype however is similar to that described in association with secondary  $\beta$ 1 laminin deficiency (Taylor *et al.*, 1997). LGMD1B and AD-EDMD have been shown to be allelic disorders following the identification of mutations in the LMNA gene among LGMD1B patients from three families (Muchir *et al.*, 2000).

However, it has also been noted that a family showing a similar phenotype to that seen in LGMD1B showed linkage to chromosome 6q23 (Messina *et al.*, 1997). The combination of autosomal dominant muscular dystrophy and cardiac disease is therefore genetically heterogeneous.

### **LGMD1C**

Mapping to chromosome 3p25, the gene product associated with LGMD1C is caveolin 3, a muscle specific component of the caveolin membrane with a probable role in signal transduction. Caveolin 3 localises to the sarcolemma, and has been shown to associate with dystrophin by immunoprecipitation experiments (Song *et al.*, 1996). Caveolin 3 has also been shown to co-immunoprecipitate with dysferlin (Matsuda *et al.*, 2001), a surface membrane protein in skeletal muscle whose deficiency causes distal and proximal, recessively inherited, forms of muscular dystrophy Miyoshi myopathy and LGMD2B respectively.

Heterozygous mutations in the caveolin 3 gene (CAV3) cause different muscle disorders, with an R27Q mutation in the CAV3 gene leading to various clinical phenotypes including hyperCKemia, rippling muscle disease, distal myopathy and LGMD1C.

Caveolin 3 labelling has been shown to be reduced in muscle from patients of two families with clinical symptoms associated with caveolin 3 mutation, including muscle cramps after exercise, calf hypertrophy and mild to moderate proximal muscle weakness. Progression of the disease was variable, and creatine kinase concentration was elevated to between 4 and 25 times normal.

### 1.3.2 THE AUTOSOMAL RECESSIVE LGMDs

To date, ten subtypes of autosomal recessive limb girdle muscular dystrophies have been classified.

## **THE SARCOGLYCANOPATHIES**

Four subtypes of LGMD – 2C, 2D, 2E and 2F, have been shown to be caused by defects in one of the sarcoglycan proteins. The sarcoglycans are a group of transmembrane proteins, component members of the dystrophin-glycoprotein complex of the muscle cell membrane, along with dystroglycan and the syntrophins (Fig 1.3).

The dystroglycans span the sarcolemma, interacting with dystrophin inside the sarcolemma and laminin in the extracellular matrix, while the syntrophins are located intracellularly. The sarcoglycans themselves are of unknown function (Worton, 1995; Straub and Campbell, 1997; Ozawa *et al.*, 1998), but because of their association with



forms of muscular dystrophy it is apparent that these proteins play a pivotal role in the maintenance of muscle membrane integrity. They all share a similar structure, having a small intracellular domain which may be located at either the C- or N-terminus, and a single transmembrane domain. The extracellular domain is large and contains potential N-glycosylation signals (Ozawa *et al.*, 1998). Expression of two of the sarcoglycans is limited to striated muscle, whilst others are more widely expressed.

Five sarcoglycan proteins have been characterised to date -  $\alpha$ -sarcoglycan,  $\beta$ -sarcoglycan,  $\gamma$ -sarcoglycan,  $\delta$ -sarcoglycan and  $\epsilon$ -sarcoglycan. The recently identified  $\epsilon$ -sarcoglycan shares similarity with  $\alpha$ -sarcoglycan and has been mapped to chromosome 7q21 (McNally *et al.*, 1998b), but unlike the other sarcoglycans has not yet been implicated in the causation of muscle disorders.

The individual sarcoglycan proteins appear to associate together as a distinct subcomplex (Yoshida *et al.*, 1994; Vainzof *et al.*, 1996; Iwata *et al.*, 1996; Inoue *et al.*, 1996; Cullen *et al.*, 1996; McNally *et al.*, 1998b). Loss of one component of the sarcoglycan complex is often associated with a secondary reduction in the expression of the others although this may not always be the case, particularly if the primary loss is of  $\alpha$ - or  $\gamma$ -sarcoglycan (Vainzof *et al.*, 1996; Jones *et al.*, 1998; Ozawa *et al.*, 1998). Loss of  $\beta$ - or  $\delta$ -sarcoglycan does however seem to be more often associated with a reduction in the expression of all the proteins in the sarcoglycan complex (Bonnemann *et al.*, 1996; Vainzof *et al.*, 1996).

A diagnosis of sarcoglycanopathy may be made by clinical examination, protein and molecular analyses, and DNA studies. Onset of the disease may be in the pelvic or shoulder girdle muscle groups, or both simultaneously. Onset of weakness in any distal, facial or extraocular muscles would tend to exclude a diagnosis of sarcoglycanopathy.

Calf hypertrophy is frequent but not always a feature, and there may be considerable intrafamilial variability. Onset of the disease may be at any age, though onset beyond the early twenties is less common. Progression of the disease may be very fast or very slow, and cardiac involvement is reported in a minority of cases. If a family history is available, it may be possible to distinguish whether the condition is dominantly or recessively inherited – dominant inheritance would be an exclusion criterion for a sarcoglycanopathy, which are all recessively inherited. Serum creatine kinase is elevated, and investigations such as muscle biopsy may show dystrophic changes. Any abnormality of dystrophin would normally be an exclusion criterion, but because of the tight association of the proteins of the dystrophin-glycoprotein complex it is now accepted that dystrophin analysis may not always be completely normal in cases of sarcoglycanopathy (Vainzof *et al.*, 1996; Jones *et al.*, 1998). The finding of muscle biopsy features diagnostic of a neuropathic process, inflammatory changes, metabolic or mitochondrial abnormalities also exclude the diagnosis. Testing of muscle biopsies with antibodies to the sarcoglycan proteins can give an indication of the level of expression. This has become the primary diagnostic tool for this group of diseases (Anderson, 1996; Sewry *et al.*, 1996). All four sarcoglycan antibodies should be used in order to identify which protein is causing the primary defect. This protein is usually completely absent while the others may be reduced, although in some sarcoglycanopathies a loss of all of the sarcoglycan proteins may be seen. Multiplex Western Blotting can also be useful (Anderson and Davison, 1999). This technique allows for comparison of the relative abundance of each sarcoglycan from a single muscle sample. From these analyses the candidate sarcoglycan gene or genes may be identified. In families containing more than

one affected or unaffected sib, haplotype analysis may be performed to aid in identification of the mutated sarcoglycan, and mutation screening performed using SSCP techniques or DNA sequencing.

The sarcoglycans are thought to maintain the structural integrity of the muscle fibre membrane, and primary defects in one of the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, or  $\delta$ -sarcoglycans have now been implicated as the cause of one of the four sarcoglycanopathies (Roberds *et al.*, 1994; Noguchi *et al.*, 1995; Lim *et al.*, 1995; Bonnemann *et al.*, 1995; Nigro *et al.*, 1996).

### **$\alpha$ -SARCOGLYCANOPATHY**

Also known as LGMD2D,  $\alpha$ -sarcoglycanopathy was previously known as 50 DAG, adhalin, A2 and SCARMD2. The protein involved in the disease is  $\alpha$ -sarcoglycan, the gene mapping to chromosome 17q12-21.33. The gene was identified after purification of  $\alpha$ -sarcoglycan, a 50kDa dystrophin associated glycoprotein (50 DAG). Cloning of the gene was possible after antibodies generated to 50 DAG were used to screen an expression skeletal muscle library. Expression of the  $\alpha$ -sarcoglycan mRNA has been shown to be within striated muscle only (Quinlivan *et al.*, 1997).  $\alpha$ -sarcoglycan has three domains – a large N-terminal glycosylated extracellular domain, a single C-terminal transmembrane domain and a short C-terminal intracellular domain. In LGMD2D patients  $\alpha$ -sarcoglycan is completely absent, and many different causative mutations have been identified across the 10 exons of the gene, which code for a cDNA of 1.474kb with an ORF of 1.161kb. These mutations include nonsense, splice site, missense and duplications, with missense mutations being the most commonly seen. Recurrent

mutations have been documented. Exon 3, which encodes part of the extracellular domain is the site most frequently showing mutations, accounting for 46% of affected chromosomes. The most frequent mutation seen is a R77C, seen on 40% of mutated chromosomes. More than 70% of patients presenting with  $\alpha$ -sarcoglycanopathy are compound heterozygotes, with cases of this form of LGMD having been reported worldwide.

$\alpha$ -sarcoglycanopathy has a variable age of onset ranging from 3-15 years, mean 8.5 years. Adult onset cases, although uncommon, have been reported, including an asymptomatic patient aged 36 years (Fanin *et al.*, 1997). Patients typically present with difficulty running and climbing stairs, some reporting muscle cramps on exercising. Toe walking can be an early feature, along with mild calf hypertrophy but occasionally involving other muscle groups. Scapular winging is often seen at presentation, although weakness tends to be in the lower limbs initially. Femoral muscles tend to be less involved than those of the pelvic group, with quadriceps and hamstrings usually showing equal involvement. Early distal lower limb involvement, if present, tends to be in the anterior tibial group. Early involvement of the deltoid is sometimes seen, with weakness of the biceps but relative preservation of the triceps. Creatine kinase levels in patients tend to be normal, or increased to the upper limit of normal. Presymptomatic elevation of creatine kinase has been reported, although is not reliable in determining carrier status.

This selectivity of muscle involvement tends to remain as the disease progresses, but eventually all muscle groups become involved apart from facial, ocular and velopharyngeal. Cardiomyopathy has been reported in some cases, and respiratory complications mean that ventilatory assistance is required. Scoliosis and contractures

may develop with progression of the disease, but no mental retardation is seen. Progression tends to be faster with earlier age of onset, patients often being confined to a wheelchair by the age of 15. However there is much variability, even intrafamiliarily, and some sufferers may continue to walk independently late into adult life.

### **β-SARCOGLYCANOPATHY**

Previously known as A3b, β-saroglycanopathy has been reclassified as LGMD2E. The β subunit of the sarcoglycan complex is a 43 kDa protein also known as 43 DAG. The protein has three domains – a large C-terminal extracellular domain, with a single transmembrane domain and a small N-terminal intracellular domain. Expression is ubiquitous. Purification of the dystrophin-glycoprotein complex led to identification of the gene, whose 6 exons code for a cDNA of approx 1kb, mapping to chromosome 4q12. Causative mutations have been identified as missense and truncating mutations. The missense mutations occur in exon 3 which codes for the immediate extracellular domain, and results in a very severe phenotype. A founder mutation has been identified in the Amish population (T151R), which results in a much milder phenotype. The contrast in the severity of the phenotypes suggests that the missense mutations may be occurring in a region of exon 3 which is particularly sensitive to their effect on the secondary structure of the protein.

Patients presenting with β-saroglycanopathy are deficient for the protein and may also display an absence or deficiency of the other sarcoglycans. Deficiency of β- and similarly δ-sarcoglycan seem often to be associated with a total deficiency of the

complex (Bonnemann *et al.*, 1996; Vainzof *et al.*, 1996). This can be detected by analysis of a muscle biopsy using specific antibodies to the various sarcoglycans. Immunostaining may also suggest which protein is primarily involved. Dystrophin levels may also be reduced but the protein is not absent, and serum creatine kinase levels are elevated.

This form of LGMD presents with an extremely broad range of severity, also showing intrafamilial variability. Amongst the Amish families studied by Lim *et al* (1995), all showing the same homozygous missense mutation, variability was such that with a mean age of onset of 7.6 years, the age at loss of ambulation ranged from 12 to 38 years (mean, 26 years). A very severe disease may occur in  $\beta$ -saroglycanopathy (Bonnemann *et al.*, 1995, 1996), with patients presenting in early childhood being confined to a wheelchair by their early teens. In contrast an Amish family heterozygous for two missense mutations presented with symptoms in adult life, and retained independent ambulation into the sixth decade (Duclos *et al.*, 1998).

As with  $\alpha$ -sarcoglycanopathy the limb and pelvic muscles are affected and there is involvement of the scapular muscles. Calf hypertrophy is also seen. Cardiac involvement and respiratory complications occur with some patients only, and sufferers display normal intellect.

### **$\gamma$ -SARCOGLYCANOPATHY**

$\gamma$ -sarcoglycanopathy, or LGMD2C, was previously termed 35 DAG, A4 and SCARMD1. It is caused by defect in the  $\gamma$  subunit of the sarcoglycan complex, which is a 35 kDa

protein having three domains – a long extracellular carboxy terminus, and single transmembrane domain and a short amino terminal cytoplasmic domain. The protein is expressed ubiquitously, and its small gene of 292 amino acids and 8 exons maps to chromosome 3q13. In cases of clinically proven  $\gamma$ -sarcoglycanopathy, the  $\gamma$ -sarcoglycan protein is completely absent from muscle biopsies. This is accompanied by a reduction or a complete absence of  $\alpha$ - and  $\beta$ -sarcoglycan, though dystrophin levels are unaffected. Serum creatine kinase levels are elevated.

Causative mutations are generally in the extracellular domain, and are either missense or more often small deletions. Founder mutations have been identified in two populations – a  $\Delta 521$ -T mutation in patients of North African and Arab origin, and a C283Y mutation in the European gypsy population.

Clinical symptoms are again similar to those of the other sarcoglycanopathies – calf hypertrophy, proximal lower limb weakness with toe walking a feature, generally no cardiac or respiratory problems and normal intelligence. Again there is much phenotypic variability, from Duchenne-like with childhood onset and loss of independent ambulation at 16-17 years, to a milder form with late onset. This variability is seen both inter- and intra-familially.

## **$\delta$ -SARCOGLYCANOPATHY**

$\delta$ -sarcoglycanopathy, or LGMD2F has causative mutations in the  $\delta$ -sarcoglycan protein. As with the other sarcoglycans, the protein has three domains – a large extracellular domain, and single transmembrane and a short intracellular domain. Its gene, mapping to

chromosome 5q33-34, has 9 exons and five mutations have been reported to date. A missense mutation E262K is present amongst Caucasians, and a founder mutation,  $\Delta$ 656C in exon 7, is present in four African-Brazilian families. This deletion results in the production of a truncated protein which causes primary deficiency of  $\delta$ -sarcoglycan in muscle tissue, and disruption of the whole sarcoglycan complex. In all cases of  $\delta$ -sarcoglycanopathy an absence of  $\alpha$ -,  $\beta$ - and  $\delta$ -sarcoglycan is seen, accompanied by an absence or reduction of  $\gamma$ -sarcoglycan. There is also a reduction of dystrophin.

The gene for  $\delta$ -sarcoglycan was identified in the Syrian cardiomyopathic hamster by candidate gene analysis. The hamster has a mutation in  $\delta$ -sarcoglycan and may manifest either a dilated or a hypertrophic cardiomyopathy, though the skeletal muscles are not prominently involved (Sakamoto *et al.*, 1997; Nigro *et al.*, 1997). Muscle from the hamster showed a deficiency of  $\alpha$ -sarcoglycan, but this was shown to be a secondary reduction from RNA analysis and mapping studies. No mutations were found in the  $\beta$ - or  $\gamma$ -sarcoglycan genes, but mutations were identified in the  $\delta$ -sarcoglycan gene. On muscle biopsy all the sarcoglycan proteins were shown to be absent, and dystrophin was present but  $\alpha$ -dystroglycan was absent or greatly reduced. The dystroglycan complex was seen to be completely disassociated from the sarcoglycan complex in the muscle cell membrane.

Families with LGMD2F showed linkage to the human region where the Syrian hamster gene causing cardiomyopathy had been mapped. These patients may potentially be at risk of cardiac complications.

Few cases of genetically proven LGMD2F have been documented so far, but those reported show a very severe clinical course (Nigro *et al.*, 1996b; Duggan *et al.*, 1997b).



Age of onset ranges from 4 to 10 years, with loss of independent ambulation at 9-16 years and death occurring at 9-19 years, usually due to respiratory failure. Patients show normal intelligence, and although muscular dystrophy is not a prominent clinical feature, muscle degeneration has been confirmed histologically.

## **LGMD2A**

Also known as calpain-deficient LGMD or calpainopathy, this form of LGMD is caused by mutations in the CAPN3 gene, which maps to chromosome 15q15.1-21.2. More than 100 pathogenic mutations have been identified to date, which are found across the entire gene as missense or nonsense substitutions, small deletions or small insertions.

The gene product, calpain 3, is a muscle specific 94-kDa calcium-activated neutral protease 3, playing indispensable roles in various cellular functions such as signal transduction, cell growth and differentiation, apoptosis and necrosis. The loss of its function leads to activation of other proteases. Calpain has also been shown to bind to titin in myofibrils (Sorimachi *et al.*, 1995), and regulates levels of I $\kappa$ B $\alpha$ , a complex involved in cell survival.

Although most of the detailed physiological functions of calpains have not yet been elucidated, their importance is apparent from the increasing number of papers reporting relationships between the malfunction of calpain and diseases such as Alzheimer's, type 2 diabetes and cataracts, as well as muscular dystrophies.

The gene responsible for LGMD2A was first localised to chromosome 15 in a population on the island of La Reunion. This highly isolated population, apparently descended from

a smaller founder population, showed a surprising heterogeneity of mutations of the CAPN3 gene. This was termed the 'Reunion paradox' (van Ommen, 1995; Richard *et al.*, 1995), and it was possible to identify six disease-associated haplotypes and subsequently mutations. In contrast, calpainopathy in other populations shows only a few recurrent mutations, one appearing to be conserved with a founder haplotype in families from such diverse geographical origins as America, Brazil and Reunion (Richard *et al.*, 1997). Common mutations have also been described in Turkey and the Basque region of Spain (Topaloglu *et al.*, 1997; Dincer *et al.*, 1997; Urtasun *et al.*, 1998).

Calpainopathy patients show normal dystrophin and sarcoglycan expression in their muscle tissue, but serum creatine kinase can be elevated to greater than 10 times normal. Muscle tissue shows evidence of apoptosis, and apoptotic myonuclei are present. Initial studies showed that calpain 3 was undetectable in normal muscle, but RNA levels were high. This led to the suggestion that the protein had a very short half-life (Sorimachi *et al.*, 1993). Subsequent studies have shown that calpain 3 is detectable in skeletal muscle (Spencer *et al.*, 1997), and a monoclonal antibody has recently been generated to calpain 3 which can be used for immunoblotting of muscle samples (Anderson *et al.*, 1998). This provides a more straightforward starting point for diagnosis than mutation detection, given the heterogeneity of the mutations seen in this large gene.

Calpainopathy patients show a predominantly symmetrical and atrophic muscle involvement, with prominent calves seen only in a minority. Contractures of the Achilles tendon may be an early feature, and contractures elsewhere, including the spine, may also be seen (C. Pollitt *et al.*, unpublished observations). Pelvic girdle weakness is evident at onset, but often with sparing of the hip abductors which may persist even with

progression of the disease, giving patients a characteristic stance. Abdominal muscles tend to be lax, and scapular winging is a common feature at onset. This symptom may be so severe that a diagnosis of scapulohumeral muscular dystrophy may be considered. Patients often present with difficulty running or climbing stairs, and may have frequent falls. The age at onset is in the majority of cases between 8 and 15 years, though a range from 2 to 40 years has been reported (Richard *et al.*, 1997). The rate of progression varies, but intrafamilial variation is less marked than that which is seen with the sarcoglycanopathies. The disease is not usually as severe as other forms of LGMD, with confinement to a wheelchair usually necessary 11-28 years after the onset of symptoms. Respiratory muscles may become involved and complications can be severe, but involvement of cardiac muscles has not been reported.

In patients who are homozygous for a mutation, genotype-phenotype correlations have been possible. As a general rule, those with homozygous null mutations tend to have the most severe clinical course.

## **LGMD2B**

Dysferlin deficient LGMD, or dysferlinopathy, is confirmed by the demonstration of mutations in the dysferlin gene, or through linkage to chromosome 2p13, to which the gene maps, in large families. The dysferlin gene encodes a novel protein which shares homology with the *Caenorhabditis-elegans* sperm vesicle fusion protein FER-1 (Achanzar, W. E. and Ward, S., 1997). Although the function of the dysferlin protein is unknown, it has been shown to be a membrane protein (Moss *et al.*, 1999; Matsuda *et al.*,

1999) which is characterised by the presence of tandem C2 domains and is predicted to have a role in membrane trafficking (Liu *et al.*, 1998; Bashir *et al.*, 1998). A possible association between dysferlin and caveolin-3 has recently been described (Matsuda *et al.*, 2001). Caveolin-3 is a skeletal muscle membrane protein important in the formation of caveolae, mutations in which cause LGMD1C. Dysferlin has been shown to co-immunoprecipitate with caveolin-3 in normal human skeletal muscle, suggesting the hypothesis that one function of dysferlin may be to interact with caveolin-3 to subserve signalling functions of caveolae (Matsuda *et al.*, 2001).

The gene for the early adult onset distal myopathy known as Miyoshi myopathy has also been shown to be localised to the same genetic interval on chromosome 2 as LGMD2B (Bejaoui *et al.*, 1995). Families linked to chromosome 2p13 with different members affected by variable phenotypes have been reported (Weiler *et al.*, 1996; Illarioshkin *et al.*, 1996). Mutations in the dysferlin gene have now been identified, and it has been shown that patients from the same family who are affected by the same homozygous mutation may present with either a limb-girdle or a Miyoshi pattern of muscle disorder (Liu *et al.*, 1998; Bashir *et al.*, 1998). It is unclear at present why dysferlin mutations should produce such phenotypic variability, but one theory which has been put forward is the existence of modifier genes of dysferlin (Anderson *et al.*, 1999; McNally *et al.*, 2000). The dysferlin gene is large, having around 55 exons, and mutations appear to be across the gene (Liu *et al.*, 1998; Bashir *et al.*, 1998). Few have been described to date but they include missense, small deletions and duplication, with a founder mutation apparent in the Libyan Jewish population.

LGMD2B patients typically present between the ages of 15-30 years after having had normal mobility in childhood. Common early symptoms include an inability to walk on tiptoe and calf atrophy. Calf hypertrophy may be present in a minority of patients early in the course of the disease, but this is only a transient feature (Bashir *et al.*, 1998). Early contractures are rare, and scapular involvement, although not present at onset, can be a mild feature with progression of the disease. The first detectable muscle weakness is often the pelvifemoral muscles, while muscle CT scanning may also show asymptomatic involvement of the gastrocnemius muscles – thus the inability to walk on tiptoe. The anterior distal leg muscles and the distal arm muscles tend to be spared, even with progression of the disease. Dystrophin, sarcoglycan and calpain 3 analyses tend to be normal, but creatine kinase levels are usually markedly elevated at presentation, often 20-150 times normal (Mahjneh *et al.*, 1996).

In contrast, Miyoshi myopathy is a predominantly distal muscular dystrophy with early involvement of the posterior lower limb muscles. As with LGMD2B, there is early involvement of the gastrocnemius muscles causing an inability to stand on tiptoes, and proximal lower and upper limb weakness often develops with progression of the disease. There is also a characteristic elevation of serum creatine kinase levels to 10-100 times normal. Having been previously described mainly in Japan (Linssen *et al.*, 1997), Miyoshi myopathy is now increasingly being recognised in the West, and it is becoming apparent that not all cases of Miyoshi myopathy map to the dysferlin gene. At least two additional Miyoshi myopathy loci have been highlighted, and a second locus has been tentatively assigned to chromosome 10 (Linssen *et al.*, 1998).

## **LGMD2G**

This form of autosomal recessive muscular dystrophy has been linked to chromosome 17q11-12. Linkage to this locus was established through the study of many large LGMD families failing to show linkage to any of the other LGMD loci (Moreira *et al.*, 1997). Causative mutations occur in the telethonin gene, whose protein product is a 19kDa sarcomeric protein localised to the Z-disc of skeletal and cardiac muscles. Telethonin has been found to be one of the substrates of the serine kinase domain of titin, and has been shown to play a role in myofibril assembly.

LGMD2G patients tend to present in their early twenties with difficulty climbing stairs and running, and foot-drop may be noted as an early feature. Proximal and distal lower limb weakness is apparent at onset, with weakness of the proximal upper limb musculature. Loss of independent ambulation is often around 18 years after onset. Muscle biopsy examination reveals normal sarcoglycan staining, but creatine kinase levels may be elevated to between 3 and 17 times normal. Histopathological studies reveal a large number of rimmed vacuoles, which are not a common feature of the autosomal recessive LGMD's. Muscle of patients with telethonin mutation displays a similar level of Z-disc disorganisation as that seen in patients with mutation in myotilin.

## **LGMD2H**

LGMD2H was first described in 1976, as a mild autosomal recessive myopathy affecting a population of Hutterites in Manitoba (Shokeir and Kobrinsky, 1976; Weiler *et al.*,

1998). The causative gene for the disease was mapped to a 6.5 Mb region in chromosomal region 9q31-33. Recent research has narrowed the candidate region to a 560 kb region on chromosome 9 containing at least four genes. Mutation screening was carried out and the gene *TRIM32*, coding for a member of the tripartite-motif (TRIM) family of proteins, was identified as the most likely gene for LGMD2H. All of the affected individuals in the study were found to be homozygous for the mutation, which replaces aspartate with asparagine at position 487, but none of the control subjects were found to have the mutation (Wrogemann *et al.*, 2002). Although the function of TRIM32 is unknown, current knowledge of the characteristic domains of this family of proteins suggest that it may be an E3-ubiquitin ligase (Joazeiro and Weissman 2000), with a function in the Ub-proteosome pathway in which proteins are assigned for degradation. This represents a novel pathogenic mechanism for muscular dystrophy.

Sufferers of LGMD2H have a variable clinical presentation (Shokeir and Kobrinsky 1976; Weiler *et al.*, 1997; Bushby 1999b). Onset is usually between the ages of 8 and 27 years, and progression of the disease is slow. Affected individuals present with proximal lower limb weakness and an elevated serum creatine kinase level (2-30 times normal). With progression of the disease, the proximal upper limb muscles become involved, predominantly the trapezius and deltoid. Mild distal limb involvement may also be seen, with weakness of the brachioradialis and anterior peroneal muscles. Most patients remain ambulatory into the sixth decade of life.

## LGMD2I

This form of LGMD was originally described in a large consanguineous Tunisian family (Driss *et al.*, 2000), where linkage to chromosome 19q13.3 was reported. This LGMD locus is identical to that of a congenital muscular dystrophy, MDC1C, suggesting that the underlying pathology of these diseases follow a similar pathway. The congenital muscular dystrophies are autosomal recessive disorders distinguishable by their age of onset which is usually within the first six months of life, with hypotonia and a dystrophic muscle biopsy. Like the limb-girdle muscular dystrophies they are highly heterogeneous. MDC1C is a severe form characterised by early onset, inability to achieve independent ambulation, muscle hypertrophy, marked elevation of serum creatine kinase, no brain involvement and a secondary deficiency of laminin  $\alpha 2$  (Mercuri *et al.*, 2001).  $\alpha$ -dystroglycan expression is also abnormal. The LGMD2I form is characterised by a high variability in clinical course, with phenotypes ranging from Duchenne-like disease course including cardiomyopathy to milder phenotypes with a slow progression. Secondary deficiencies of  $\alpha$ -dystroglycan and  $\alpha 2$ -laminin are also a feature.

The causative mutation is in the fukutin-related protein gene (FKRP), which shares sequence similarities with a family of proteins involved in the glycosylation of cell surface molecules (Brockington *et al.*, 2001b). Part of our work has been in this area, and has led to the identification of mutations in the same gene amongst affected individuals from 17 LGMD2I families. LGMD2I has therefore been shown to be a milder allelic variant of congenital muscular dystrophy MDC1C.



The most common mutation in FKRP, which encodes a putative glycosyltransferase, is C826A (Leu276Ile). If homozygous this results in a clinically less severe LGMD2I phenotype, but if heterozygous the disease runs a much more severe clinical course. A missense mutation in the FKRP gene results in an MDC1C phenotype, suggesting that the C826A change is a less disruptive FKRP mutation.

## **MDC & LGMD2I**

Congenital muscular dystrophy is an autosomal recessive muscular dystrophy with early onset (Voit, 1998). Sufferers show generalised hypotonia, with contractures and joint deformities, and histologically, patient muscle shows dystrophic changes as well as white matter changes in the brain. There are several forms of congenital muscular dystrophy, including MDC1A, MDC1B and MDC1C. These disorders also share clinical features with three other myopathies, Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (Emery, AEH., ed. Neuromuscular disorders: Clinical and Molecular Genetics (Wiley, 1998) pp. 21-48). The congenital muscular dystrophies are divided into two groups, merosin-deficient CMD and merosin-positive CMD. Mutation in the merosin gene causes complete loss of the protein, resulting in a severe muscular dystrophy phenotype (Voit, 1998). Sufferers show severe neo-natal hypotonia and delayed motor development. Atrophy and weakness of muscles are a feature, as well as respiratory problems. Serum creatine kinase levels are shown to be elevated, and examination of the brain shows changes in white matter. In contrast, with non-merosin linked CMD the presence of the protein

merosin results in a milder clinical course (Voit, 1998). Patients still show the same symptoms of neo-natal hypotonia, delayed motor development, muscle atrophy and weakness and respiratory problems along with elevated creatine kinase levels, but there are no changes in the white matter of the brain. Not all merosin deficient CMD are due to mutations in the merosin gene, suggesting that merosin deficiency is a secondary feature. MDC1C shows merosin deficiency but maps to chromosome 19q13.3 and is due to mutation in the FKRP gene (Brockington *et al.*, 2001b).

Merosin ( $\alpha$ -laminin) is a muscle specific laminin, existing in the extracellular domain where it forms a major component of the basement membrane (Emery, AEH., ed. Neuromuscular disorders: Clinical and Molecular Genetics (Wiley, 1998) pp. 21-48).

Three different laminin chains,  $\alpha$ ,  $\beta$  and  $\gamma$ , exist together in different combinations to form up to 11 laminin isoforms, of which the  $\alpha 2$  chain is involved in the muscle cell membrane. This chain links with a receptor on the  $\alpha$ -dystroglycan component of the dystrophin-glycoprotein complex (Emery, AEH., ed. Neuromuscular disorders: Clinical and Molecular Genetics (Wiley, 1998) pp. 21-48).

Congenital muscular dystrophy type 1A, mapped to chromosome 6q22-23, is due to mutations in the LAMA2 gene causing deficiency of merosin (Hebling-Leclerc *et al.*, 1995). Other CMD genes include the fukutin related protein gene FKRP, encoding a protein which, as suggested by its name, is related to fukutin. FKRP is also a putative glycosyltransferase. Mutations in the FKRP gene cause both limb-girdle muscular dystrophy type 2I (LGMD2I), and congenital muscular dystrophy type 1C (MDC1C) (Brockington *et al.*, 2001a). The mutation C826A (Leu276Ile) in the FKRP gene is the most common mutation found in any autosomal recessive muscular dystrophy in the UK

(Brockington *et al.*, 2001a). If present homozygously, the mutation results in a milder LGMD2I phenotype, but if inherited heterozygously the disease runs a much more severe clinical course. Missense mutations in the FKRP gene are associated with the MDC1C phenotype, but no null mutations are observed in these patients, suggesting that these mutations are embryonic lethal.

The fukutin and fukutin-related protein genes encoding putative glycosyltransferases are involved in the glycosylation of dystroglycan (Martin-Rendon and Blake, 2003).  $\alpha$ -Dystroglycan is a receptor for laminin and this has been shown by analysis of patient muscle by Western blotting using  $\alpha$ -dystroglycan antibodies. If not glycosylated correctly, dystroglycan fails to bind laminin, altering the stability and architecture of the muscle cell. LGMD2I and MDC1C patients both show abnormal glycosylation of  $\alpha$ -dystroglycan (Martin-Rendon and Blake, 2003). In these studies  $\alpha$ -dystroglycan is absent or reduced and shows altered mobility of SDS gels.

Protein glycosylation occurs in the endoplasmic reticulum and the Golgi apparatus. Proteins with roles in cell-cell adhesion have been shown to be heavily glycosylated. Proteins can be grouped into N-glycans and O-glycans depending upon the amino acid residue which is glycosylated (Martin-Rendon and Blake, 2003). O-glycosylation involves serine and threonine residues, N-glycosylation involving asparagine residues (Paulson, 1989). The different sugars are added to proteins and they impact on the structure and function of the protein (Breton and Imberty, 1999). O-mannose linked glycosylation is high in the brain, peripheral nerve and muscle tissue, with 30% of O-linked sugar chains in the brain being O-mannose (Van den Steen, *et al.*, 1998).  $\alpha$ -

Dystroglycan is an O-mannose linked protein, which goes some way towards explaining the occurrence of white matter changes in the brains of CMD patients.

## **1.4 MULTIPLE MECHANISMS CAUSING MUSCULAR DYSTROPHY**

The mapping of the muscular dystrophies to so many chromosomes, and the identification of multiple causative mutations has served to highlight the different mechanisms by which muscular dystrophy can arise, with three distinct mechanisms now emerging.

### **1.4.1 DYSTROPHIN/GLYCOPROTEIN COMPLEX**

The dystrophin/glycoprotein complex forms a bridge across the muscle membrane, between the inner cytoskeleton (dystrophin) and the basal lamina (merosin). It is thought to provide structural integrity to the muscle cell membrane, protecting muscle fibres from long term contraction-induced damage and necrosis.

Mutations causing loss of most of the dystrophin from the muscle cell membrane usually result in the Duchenne phenotype of muscular dystrophy. Mutations generating a larger or smaller dystrophin molecule but not resulting in its complete loss usually result in the milder Becker phenotype.

#### 1.4.2 TITIN/SARCOMERIC PROTEINS

Titin is a giant muscle protein (363 exons), stretching from the Z disc to the M line. It provides muscle elasticity and acts as a docking bay for the sarcomeric proteins involved in muscle contraction. Four muscular dystrophies have been linked to defects in these proteins: LGMD1A, mapping to chromosome 5q and showing mutation in the gene product myotilin; LGMD2A, mapping to chromosome 15q with defects in the protein calpain 3; LGMD2G, mapping to chromosome 17q and showing mutation in the sarcomeric protein telethonin; and TMD, mapping to chromosome 2q with defects in the protein titin.

#### 1.4.3 ABNORMAL GLYCOSYLATION

Abnormal glycosylation of  $\alpha$ -dystroglycan or laminin  $\alpha$ 2 is seen in many muscular dystrophies including MDC1C, FCMD, MEB, Walker-Warburg and also LGMD2I, in which patients show reduction or deficiency of  $\alpha$ -dystroglycan.  $\alpha$ 2-laminin is a constituent of the basal lamina which links to dystroglycan and which provides structural support in the extracellular matrix. Mutations in genes with glucosyltransferase activity have been identified as responsible for these diseases, suggesting that abnormal processing of  $\alpha$ -dystroglycan may be central to the pathogenesis of a significant number of genetic conditions (Muntoni *et al.*, 2002).

## **1.5 AIMS**

The aims of this study were two-fold.

### **1. To perform genetic analysis of the LGMD2I region on chromosome 19q13.3.**

- a) In a collection of 6 unclassified LGMD families, haplotypes of chromosome 19q13.3 were generated to identify potential LGMD2I families.
- b) In parallel the genome databases were screened to assemble a physical and a transcript map of the LGMD2I region.
- c) From this map candidate genes were identified for mutation screening.

### **2. To perform genetic analysis of non-dysferlin Miyoshi myopathy.**

- a) In 2 large non-dysferlin MM families haplotypes of chromosome 10p were generated. Recombinant and non-recombinant regions were identified and compared between affecteds in the two families. This data, together with the evidence of tentative linkage to chromosome 10p in these families was used to determine the existence of the MMD2 gene.
- b) In 7 additional non-dysferlin MM families haplotypes for chromosome 10 and chromosome 3, to which caveolin 3 maps, were generated, to provide insight into the genetic heterogeneity of non-dysferlin MM and the role of caveolin 3 in non-dysferlin MM.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

Unless specified otherwise, all inorganic chemicals were purchased from Merck, BDH Laboratory Supplies, Poole, BH15 1TD, England.

## **2.1 FAMILY INFORMATION**

Genomic DNA from fifteen clinically assessed families were used, after ethical consent was given for their use in this study by the local MREC and Durham University ethical committee.

### **2.1.1 PATIENT DNA SAMPLES**

In order to preserve anonymity, the samples were assigned a code upon receipt for use during this study. The families themselves were numbered from one to fifteen, and individual members of the family were assigned P for a parent or C for a child. If more than one child was present in the family, then C1 denotes the first child, C2 the second and so on. M or F then indicates whether the individual is male or female, and finally A denotes an individual affected by muscular dystrophy and U an individual unaffected. Thus 5-C3/FA is the third child of family 5, who is a female affected by muscular dystrophy. In the case of Family 15, DNA from four further related family members was introduced. These individuals were assigned R for relative. This information is summarised in Table 2.1. Pedigrees for the fifteen families are shown in the Appendix.



Table 2.1 DNA Samples

Family Number	Family Member	Stock Tube Reference	Stock conc. ng/ul	New Reference
1	Parent, male	236	300	1-P/MU
	Parent, female	237	200	1-P/FU
	Child, female	238	200	1-C1/FA
	Child, male	235	300	1-C2/MA
2	Parent, female	25	200	2-P/FU
	Parent, male	24	<10	2-P/MU
	Child, male	26	200	2-C1/MA
	Child, female	27	200	2-C2/FA
	Child, female	29	50	2-C3/FU
3	Parent, female	7-167	200	3-P/FU
	Child, male	7-169	200	3-C1/MU
	Child, male	7-170	150	3-C2/MU
	Child, male	7-171	200	3-C3/MA
	Child, female	7-172	300	3-C4/FA
4	Parent, male	1.1569	300	4-P/MU
	Child, male	1.157	300	4-C1/MU
	Child, male	1.1571	300	4-C2/MU
	Child, female	1.1524	100	4-C3/FA
	Child, male	1.1716	250	4-C4/MU
	Child, male	1.1651	250	4-C5/MU
	Child, male	1.1715	300	4-C6/MU
	Child, female	1.1717	250	4-C7/FU
	Child, female	1.1523	400	4-C8/FA
	Child, male	1.1718	150	4-C9/MU
5	Parent, male	5.632	400	5-P/MU
	Child, male	5.634	400	5-C1/MU
	Child, female	5.633	400	5-C2/FU
	Child, female	10.197	400	5-C3/FA
	Child, male	5.568	400	5-C4/MA
6	Parent, male	5.144	400	6-P/MU
	Parent, female	5.143	400	6-P/FU
	Child, female	5.142	400	6-C1/FA
	Child, female	5.141	400	6-C2/FA
7	Child, male	A	2000	7-C1/MU
	Child, female	B	2000	7-C2/FU
	Child, female	C	2000	7-C3/FU
	Child, female	D	200	7-C4/FU
	Child, female	E	2000	7-C5/FU
	Child, male	F	2000	7-C6/MU
	Child, male	G	2000	7-C7/MA
	Child, female	H	2000	7-C8/FA
8	Child, female	I	200	8-C1/FU
	Child, female	J	1000	8-C2/FU
	Child, female	K	1000	8-C3/FU
	Child, female	L	1000	8-C4/FU
	Child, male	M	1000	8-C5/MU
	Child, female	N	1000	8-C6/FA
	Child, male	O	1000	8-C7/MA

Family Number	Family Member	Stock Tube Reference	Stock conc. ng/ul	New Reference
9	Parent, male	5.4701	200	9-P/MU
	Parent, female	5.47	200	9-P/FU
	Child, female	5.4681	200	9-C1/FU
	Child, female	5.4682	200	9-C2/FA
10	Parent, female	1.1193	200	10-P/FU
	Child, male	1.11928	200	10-C1/MU
	Child, male	5.56	200	10-C2/MA
	Child, male	1.11929	200	10-C3/MU
11	Parent, male	5.1383	200	11-P/MU
	Parent, female	5.138	200	11-P/FU
	Child, female	5.1379	200	11-C1/FU
	Child, female	5.1382	200	11-C2/FU
	Child, female	5.136	200	11-C3/FA
	Child, male	5.1384	200	11-C4/MU
	Child, male	5.1405	200	11-C5/MU
	Child, male	5.1381	200	11-C6/MU
	Child, male	5.1361	200	11-C7/MA
12	Parent, female	5.2397	200	12-P/FU
	Child, male	5.2396	200	12-C1/MU
	Child, male	5.2395	200	12-C2/MU
	Child, male	5.2180	200	12-C3/MA
13	Parent, male	5.4211	200	13-P/MU
	Parent, female	5.4153	200	13-P/FU
	Child, female	5.4258	200	13-C1/FU
	Child, male	5.4154	200	13-C2/MA
	Child, female	5.4242	200	13-C3/FU
	Child, female	5.4243	200	13-C4/FU
	Child, female	5.4155	200	13-C5/FA
14	Parent, male	5.373	200	14-P/MU
	Parent, female	5.3376	200	14-P/FU
	Child, female	5.3552	200	14-C1/FU
	Child, female	5.3465	200	14-C2/FU
	Child, female	5.3375	200	14-C3/FA
15	Child, male	8	10000	15-C6/MA
	Child, male	9	10000	15-C4/MA
	Parent, male	10	10000	15-P/MU
	Parent, female	11	10000	15-P/FU
	Relative, male	12	10000	15-R1/MU
	Relative, female	13	10000	15-R2/FU
	Relative, female	14	10000	15-R3/FU
	Relative, male	15	10000	15-R4/MU
	Child, female	16	10000	15-C1/FU
	Child, male	17	10000	15-C2/MU
	Child, female	18	10000	15-C3/FU
	Child, female	19	10000	15-C5/FU

## **2.2 AGAROSE GEL ELECTROPHORESIS**

DNA was electrophoresed on horizontal slab minigels (50-75ml volume) ranging in concentration from 0.8-1.5% (w/v) (0.8-1.5% agarose (BMA), 10% 10X TAE buffer, 0.5µg/ml ethidium bromide (Sigma)). Electrophoresis was performed in 1X TAE buffer (40mM Tris, 1mM EDTA, pH 7.7) containing 0.2µg/ml ethidium bromide, at 100V for 10-40 minutes. The DNA within the gel was visualised under long wave ultraviolet light using a Gel Doc 1000 transilluminator (BIO RAD).

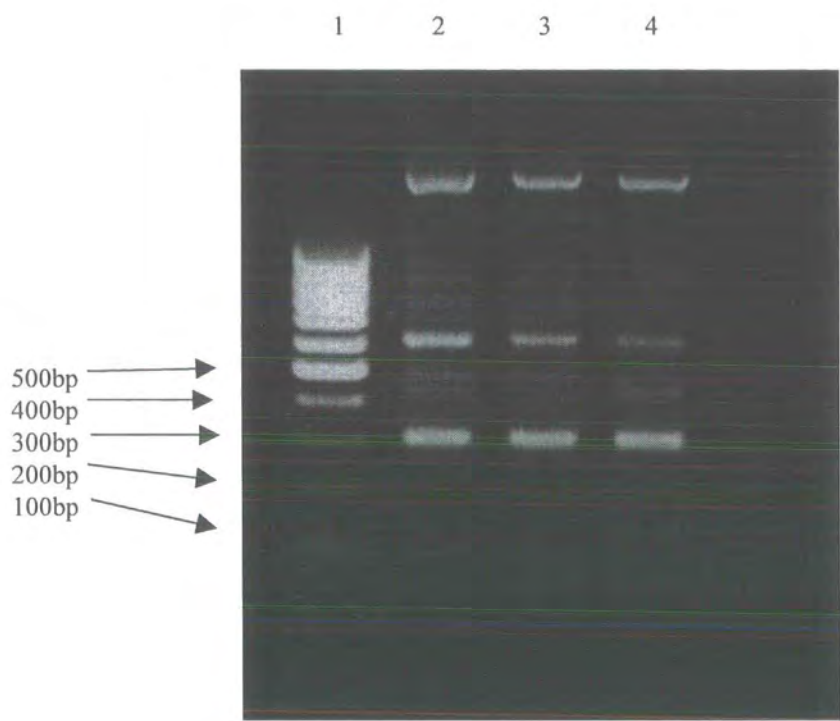
Genomic DNA was quantified by loading 1µl of DNA combined with 9µl of loading buffer (31.25% glycerol, 10mM Tris pH8.0, 0.01M EDTA pH8.0, 1mg/ml Fast Orange G (Sigma)), against a 200ng and 100ng standard (pGEM® 3Zf(+), Applied Biosystems). An estimation of the concentration of each sample was recorded (see Table 2.1), and the stock DNA samples were then diluted to a working concentration of 100pmol/µl.

PCR reactions for genotyping were prepared for electrophoresis by adding 5µl of sample to 5µl of loading buffer. Samples were run against 5µl of GeneRuler™ 100bp DNA Ladder (MBI Fermentas), facilitating the identification and quantification of the required PCR bands. An example is shown in Fig. 2.1.

PCR reactions for SSCP analysis were prepared as above for genotyping, and quantified by comparison to 5µl of GeneRuler™ 100bp DNA Ladder (MBI Fermentas).

**Fig. 2.1 Agarose gel containing completed PCR reactions run against 100bp ladder.**

PCR products were generated using microsatellite marker D10S570. The required reaction product, 287-305bp, was identified by comparison to the 100bp ladder in lane 1.



### 2.3 MICROSATELLITE ANALYSIS

The LGMD2I locus maps to chromosome 19q13.3. Genetic data suggests that the mutated gene lies between the microsatellite markers D19S606 and D19S879 (Driss *et al*, 2000). Microsatellite markers mapping within this region were analysed in families 1-6. Chromosome 19 markers are described in Table 2.2.

A second locus for Miyoshi Myopathy (MM) has been tentatively assigned to chromosome 10p (Linssen *et al.*, 1998). Families 7-15 were analysed initially using chromosome 10 markers identified by Linssen and colleagues, then informative families were analysed further using markers mapping distally and proximally to this region. Chromosome 10 markers are described in Table 2.2.

Primers used for genotyping were labelled at the 5' end with a fluorescent dye, either 6-FAM, NED or HEX (NED – Applied Biosystems, 6-FAM, HEX – Thermo Hybaid, MWG-Biotech, Invitrogen). Details of the primer sequences and PCR product sizes are given in table 2.2.

PCR amplification of microsatellite loci under standard conditions was performed in 25µl volumes in PCR buffer (10% 10X buffer (supplied with Taq enzyme (Promega))) containing 2.5mM each dNTP in the presence of 1.5mM MgCl<sub>2</sub>. One unit of Taq enzyme (Promega) was used for DNA synthesis. Genomic DNA was present at a concentration of 100pmol/µl with forward and reverse oligonucleotides at 20µM.

PCR conditions employed were: denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min. Reactions were carried out on a DNA thermal cycler (Perkin Elmer).

Marker	Forward primer sequence 5' → 3'	Reverse primer sequence 5' → 3'	Modification	PCR product size (bp)
D19S596	GAA TCC GAG AGG TGG G	GCC AGA GCC ACT GTG T	5': 6-FAM	213-221
D19S902	CCA TCC TAA TGA GGG CAA	GCA CCA GTG ACT GCC TGT	5': NED	199-217
D19S606	AGG GCT GGG ACC TCA C	CCA ACA CAC TGT CTG CCT T	5': 6-FAM	172-190
D19S879	CTG AGT GTG AAT GAG GCA AC	AGG CCA GAG GAC TGA TTG	5': HEX	217-265
D10S1745	CCC ACA TGG CAG GAT TC	ATA ACC CAT AAT AGC CAA GAT ACG G	5': 6-FAM	149-175
D10S1713	GAC AGC AAC TAA CCT CCT GTA AG	TGT GTT ATT CAA GGG TCA GC	5': 6-FAM	245-255
D10S591	ACC TCG AAG GTC TGT TCT CC	GGC TTT ATG GAT CAT ATT AAT CCA C	5': HEX	212-232
D10S553	AAG GTT ACT GAG AAG ACA GTT TTC A	GGG TGC TAG TCC AGA CAT TT	5': 6-FAM	205-235
D10S2194	CAA TTT GGA AAA CTT TTA TCA ATC A	TTT GAG ATG GGT CTT ACA ATT GG	5': NED	226-227
D10S1674	AAC AGA AGT CAA ATC AAT GAA C	ACT AGA GGC GCC CAC C	5': 6-FAM	163-193
D10S1768	AGA AGC CGT GTC TGC C	CCC AGG GAC TTA GGG TG	5': HEX	163-181
D10S1666	CAT GGA CCC ATC GGT G	GCT GGT CTC AAA CCT CCC	5': 6-FAM	218-277
D10S191	CTT TAA TTG CCC TGT CTT C	TTA ATT CGA CCA CTT CCC	5': 6-FAM	124-152
D10S547	CTT GAA AGG CGG AGG C	CCC AAT AGT CCA CAG GGA G	5': 6-FAM	235-250
D10S570	GCA TTC ATC CAA CAA GCA TA	AAT TAG TTC CAT GGG CAC AG	5': 6-FAM	287-305
D10S2325	CTC ACG AAA GAA GCC TTC TG	GAG CTG AGA GAT CAC GCA CT	5': 6-FAM	119-154
D10S1734	GCC TGG GTG ACA GAG TGA GAT TCT A	ACA CAC GTA CACATG GGG TGG T	5': 6-FAM	163-189
D3S3611	GCT ACC TCT GCT GAG CAT	TAG CAA GAC TGT TGG GG	5': 6-FAM	107-137
D3S3691	TCT CAG CAA TAG CAA ACA TCA GG	TTG AAA CCA GGG TGA CAA ATA CAT C	5': 6-FAM	214-260
D3S1307	TAA ATG ACA CTC CAG CAG CA	GCA CTC ATC AAT GTA TGG GG	5': HEX	237-251
D3S1270	TGG AAC TGT ATC AAA GGC TC	TTG CAT TAG AGC TAT TCT CCA GA	5': 6-FAM	164-186

Table 2.2 Microsatellite Markers

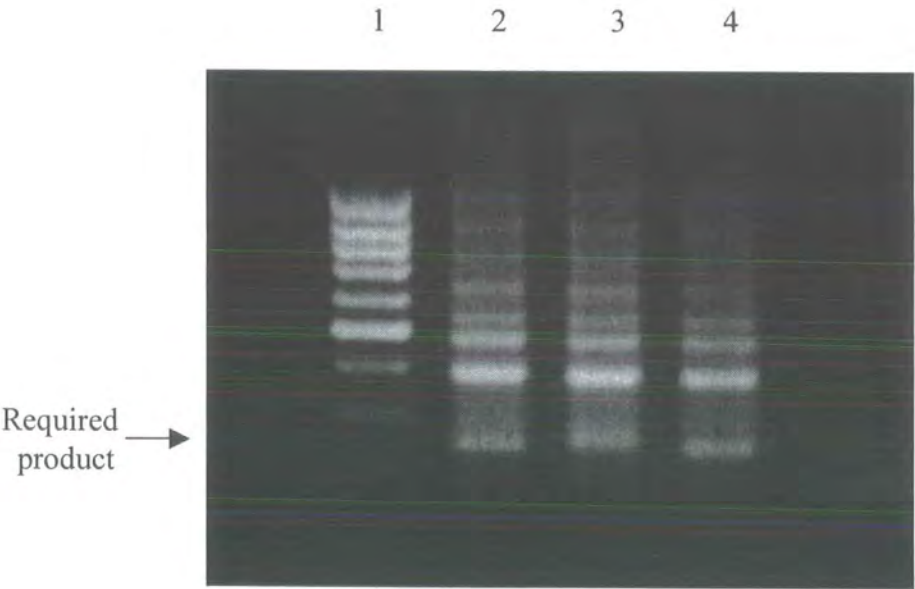
In some cases the standard PCR conditions produced many non-specific products (Fig. 2.2), or the yield of the required product was very low. If the non-specific products were of a similar size to the required product, then the PCR reaction was repeated under optimised conditions in order to reduce the generation of the non-specific products which interfere with accurate sizing of alleles during genotyping. Optimisation was also carried out to improve yield of the required product where necessary. This involved varying the concentration of  $\text{MgCl}_2$  between 1.5mM and 2.5mM, and the concentration of the primers between 15 $\mu\text{M}$  and 25 $\mu\text{M}$ . In some cases it was also necessary to vary the annealing temperature between 50°C and 55°C. Fig. 2.3 shows PCR products generated after optimisation was carried out to reduce the generation of non-specific products.

## **2.4 GENOTYPING**

Genotyping was carried out on an ABI Prism 377XL DNA Sequencer. Data is collected by automated fluorescence detection using the software Data Collection v2.5 (Applied Biosystems). Using this system, DNA fragments can be labelled with up to three different coloured fluorescent dyes, a fourth dye colour is reserved for labelling an internal size standard. By electrophoresing the size standard simultaneously with each individual sample, precise size calling is performed without the problems of band-shift artifacts or run-to-run variation. The software GeneScan® Analysis v3.1 (Applied Biosystems) analyses the data collected to accurately size DNA fragment molecular lengths based on their normalised mobilities. The software uses the size standard to create a sizing curve for each lane, then determines the length of each dye-labelled PCR

**Fig. 2.2 Agarose gel containing completed PCR reactions showing non-specific extension products**

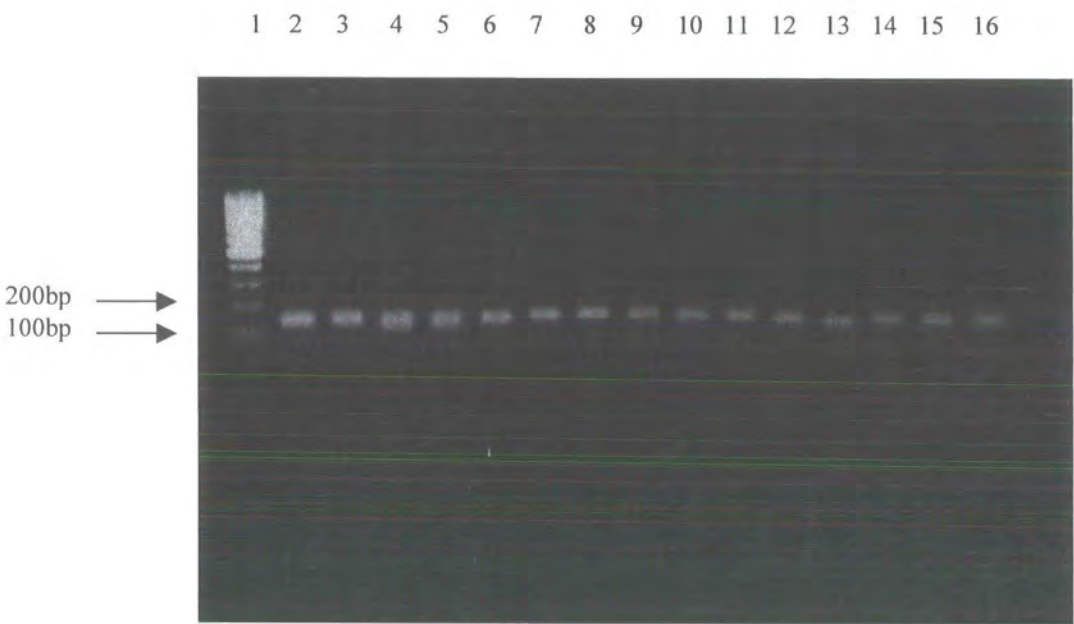
The samples in lanes 2, 3 and 4 show many non-specific extension products and smearing. The required product, indicated below, is of a low concentration compared to the non-specific products.





**Fig. 2.3 Agarose gel containing completed PCR reactions after optimisation.**

PCR products were generated using microsatellite marker D10S191. The required product, 124-152bp, is indentifiable by comparison to the 100bp ladder in lane 1 and the generation of non-specific reaction products has been eliminated.



product by comparing it with the sizing curve for the specific lane in which it was electrophoresed. This allows resolution of up to 1 base pair, which is necessary for the analysis of microsatellites or short tandem repeats (STRs).

For genotyping, PCR amplified DNA was used at a concentration of 10-20ng/μl. This was combined with GeneScan™ 500 ROX™ Size Standard (Applied Biosystems) in loading buffer (0.2-0.4μl labelled PCR product, 0.125μl ROX, 1.5μl loading buffer (deionised formamide containing 10% Blue dextran/EDTA (Applied Biosystems))).

Because each of the fluorescent dyes used (6-FAM, NED, HEX) has a different absorption maximum, the intensity of fluorescent signal emitted differs for each dye. Therefore in order to generate signals of equal intensity for each dye during data collection, it was necessary to load greater amounts of PCR products labelled with low signal intensity emission dyes, compared to those with high signal intensity emission dyes. Hence the amount of labelled PCR product loaded onto the gel varied from 0.2-0.4μl.

It was possible to multiplex samples into a single lane by using either colour or fragment length to distinguish between the fragments. Hence PCR products of similar lengths were electrophoresed simultaneously in one lane provided the primers were labelled with different colours, and products with the same fluorescent labels could be combined if their product lengths were different. These considerations formed the criteria for selection of fluorescent labels for each individual marker used. When multiplexing was carried out, the appropriate volume of each PCR product was pooled and then combined with 0.125μl of ROX and 1.5μl of loading buffer.

Samples were electrophoresed on a 4% denaturing polyacrylamide 36cm WTR slab gel (6M urea, 4.5% acrylamide/bis-acrylamide, 29:1 ratio, 10% 10X TBE, 0.05% ammonium persulfate, 0.05% TEMED) in 1X TBE buffer (0.09M Tris, 0.09M orthoboric acid, 2mM EDTA), using a 50-lane square tooth comb. Electrophoresis was carried out at 3kV for 3 hours, using virtual filter set D. These parameters were selected in order to achieve high resolution (up to 1bp) rather than high speed analysis.

The collected data was analysed initially using GeneScan® Analysis v3.1 (Applied Biosystems). The files were then imported into Genotyper® v2.5 (Applied Biosystems) for further analysis, where the detected peaks were sized. Fig 2.4 shows an example of a Genotyper® file.

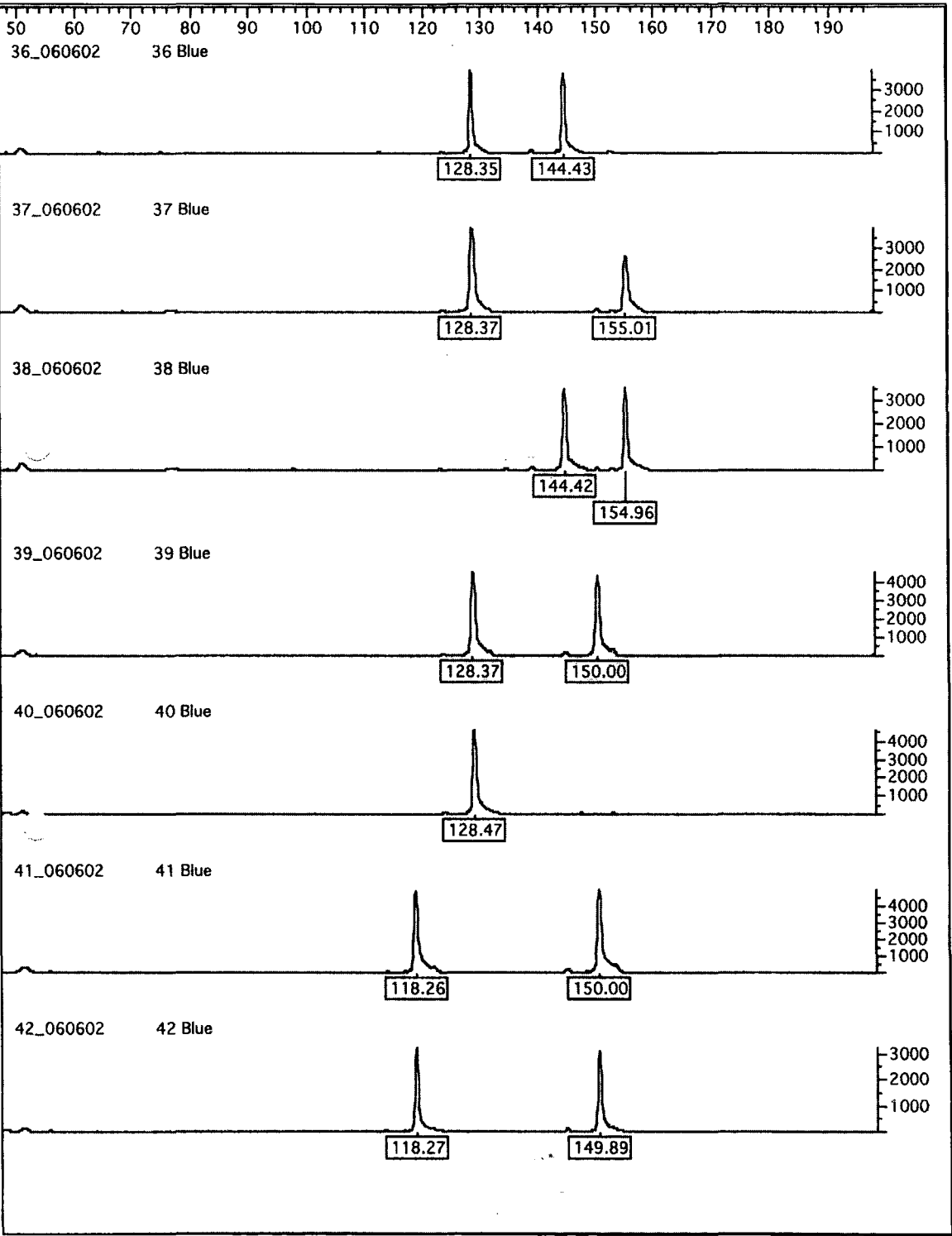
## **2.5 BIOINFORMATICS**

### **2.5.1 MOLECULAR ANALYSIS OF CHROMOSOME 19**

LGMD2I was mapped to 19q13.3 by Driss *et al*, (2000). The microsatellite markers described in this paper were used in this study to determine whether the haplotypes of families 1-6 were consistent with linkage. The primer sequences of these markers were found by accessing the UniSTS database, through the NCBI (National Center for Biotechnology Information) website, whose home page is at <http://www.ncbi.nlm.nih.gov>. The UniSTS database is a NCBI resource that reports information about markers, or Sequence Tagged Sites (STS). Marker and mapping data is integrated from public resources including GenBank, RHdb, GDB and various human

**Fig. 2.4 Genotyper® file**

Genotyper® file showing PCR products generated using the microsatellite marker D10S2325. Accurate sizes in base pairs are given in boxes below each peak. This file shows five alleles in one family studied – 118bp, 128bp, 144bp, 150bp and 155bp.



and mouse maps. Information about primer sequences, product size, and mapping information can be obtained, and links to other databases are available. Fig. 2.5 shows a UniSTS entry for the microsatellite marker D19S596.

The Entrez database was screened in order to locate the genomic contigs with which each of the microsatellite markers are associated. Entrez is a text-based search and retrieval system which covers all the major databases at NCBI, including PubMed, Nucleotide and Protein Sequences, Protein Structures, Complete Genomes, Taxonomy, OMIM, and others. Entrez is accessible at <http://www.ncbi.nih.gov/Entrez/>. The database BLAST (Basic Local Alignment Search Tool) was also screened independently to confirm the data. BLAST provides a method for rapid searching of nucleotide and protein databases, and is accessible at <http://www.ncbi.nlm.nih.gov/BLAST/>. Fig 2.6 shows the result of an Entrez search for the microsatellite marker D19S596. The first entry shows the contig address, NT\_011109.

The NCBI Map Viewer was used to find gene information by position relative to other landmarks. Map Viewer presents genetic information for many genomes, and the availability of whole genome sequences means that objects such as genes, markers, clones, sites of variation and clone boundaries can be positioned by aligning defining sequence from these objects against the whole genomic sequence. This position information can then be compared to information from other genetic or physical mapping. Using this resource, the order of the markers and their contig assignment was established. By zooming in on the map it was possible to obtain Gene Sequence information (provisional gene order). Fig 2.7 shows a fully detailed view within Map Viewer, showing the gene FLJ10922 and its position on the contig NT\_011109.

Fig. 2.5 UniSTS

Results of search for microsatellite marker D19S596, showing forward and reverse primer sequences and PCR product size.

NCBI

PubMedEntrezBLASTOMIMTaxonomyStructure

SearchUniSTSforGo

Entrez UniSTS  
Help/FAQ  
Query tips  
Submit  
Submit Maps  
FTP site  
Statistics  
Related sites  
e-PCR  
Map Viewer  
LocusLink  
UniGene  
dbSNP  
GeneMap'99  
RHdb  
GDB  
MGD  
ZFIN  
Genome  
Resources  
H. sapiens  
M. musculus  
R. norvegicus  
D. rerio

D19S596UniSTS:15315

Primer Information

Forward primer:AGGGCTGGGACCTCAC  
Reverse primer:CCACACACTGTCTGCCTT  
PCR product size:172-190 (bp), Homo sapiens  
GenBank Accession:Z33152

Homo sapiens

Name: D19S596  
Also D19S606, Z33152, B030WE1, AFMa133zh9, AFMb030we1, stSG34742,  
known as: RH74437, RH84008, RH86248, W6232, HSB030WE1, SHGC-21097


Cross References

SNP	rs2245418	Summary
RH details	RH74437	Genebridge4
	RH84008	Genebridge4
	RH84008	Stanford G3
	RH86248	Genebridge4
	RH86248	Stanford TNG
GDB	GDB:378151	

Mapping Information

Fig. 2.6 Entrez Nucleotide

Results of Entrez Nucleotide search for D19S596. The first entry shows the contig address NT\_011109.



PubMed

Nucleotide

Protein

Genome

Structure

PMC

Taxonomy

Search

Nucleotide

for

D19S596

Go

Clear

Limits

Preview/Index

History

Clipboard

Details

Display

Summary

Show

20

Send to

Text

Items 1-2 of 2

☐ 1: [NT\\_011109](#)

Homo sapiens chromosome 19 genomic contig  
gi|29800594|ref|NT\_011109.1|Hs19\_11266[29800594]

☐ 2: [Z52219](#)

H.sapiens (D19S596) DNA segment containing (CA) repeat, clone AFMa133zh9, single read, sequence tagged site  
gi|1233519|emb|Z52219.1|HSA133ZH9[1233519]

About Entrez

Search for Genes

Entrez Nucleotide

Batch Entrez

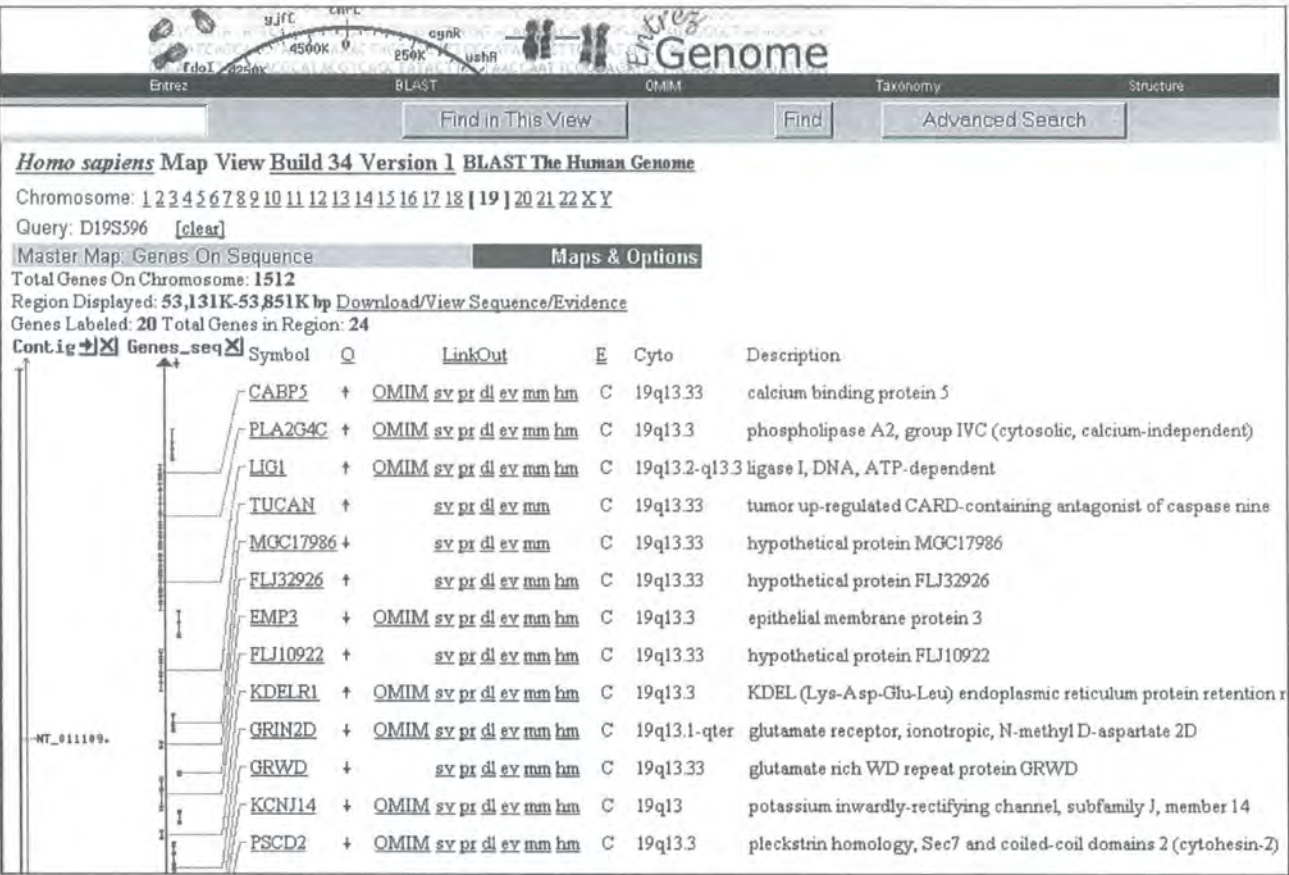
Check sequence revision history

How to create WWW links to Entrez

LinkOut

Fig. 2.7 Map Viewer

Map viewer resource within Entrez Genome, showing a section of the contig NT\_011109 and its respective genes.





In order to identify candidate genes for analysis, the resource LocusLink was used. LocusLink organizes information from collaborating public databases and from other groups within NCBI to provide a locus-centred view of genomic information. Each LocusLink entry contains a collection of links to provide more information about a locus. The resource is accessible at <http://www.ncbi.nlm.nih.gov/LocusLink/>, or can be accessed by clicking on the genes of interest displayed within the contigs in Map Viewer. Fig. 2.8 shows a LocusLink entry for the gene FLJ10922. Each of the genes were screened in LocusLink, and their entries in UniGene and OMIM were also viewed. UniGene partitions GenBank sequences into a non-redundant set of gene-oriented clusters, each of which contains sequences that represent a unique gene, as well as related information such as the tissue types in which the gene has been expressed and map location. This resource can be accessed via LocusLink, or directly at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene>. Fig. 2.9 shows the UniGene entry for the gene FLJ10922. OMIM (Online Mendelian Inheritance in Man) is a directory of human genes and genetic disorders. It too is accessible via LocusLink, or directly at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>. OMIM provides text overviews of genetic disorders and gene loci, and provides connections to other data resources such as bibliographic, sequence and map information. Entries may also include clinical features, inheritance, population genetics, heterogeneity and mapping information. With reference to LocusLink, UniGene and OMIM a profile was built for each gene, including mapping data, characterization, expression information and any published information available. From this data it was possible to identify candidate genes for mutation screening.

Fig. 2.8 LocusLink

Results of LocusLink search for the gene FLJ10922 showing the hypothetical protein product FLJ10922 with unknown function.

NCBI

LocusLink

PubMedEntrezBLASTOMIMMap ViewerTaxonomyStructure

Search: LocusLinkDisplay: BriefOrganism: All

Query:GoClear

View: Hs FLJ10922One of 1 LociSave All Loci

A B C D E F G H I J K L M N O P Q R S T U V W X Y Z

Click to Display mRNA-Genomic Alignments (spanning 31,570 bps)

PUBACEVIEWUNIGENEMAPVARHOMOLefUCSC

MGC

Homo sapiens Official Gene Symbol and Name

None Available

Interim Gene Symbol and Name:

FLJ10922: hypothetical protein FLJ10922

LocusID: 55260

OverviewSubmit GeneRIF?

Locus Type:gene with protein product, function unknown

Product:hypothetical protein FLJ10922

Map Information?

Chromosome:19mv

Cytogenetic:19q13.3RefSeq

Markers:Chr. 19RH102358mv

NCBI Reference Sequences (RefSeq)?

Category: PREDICTED

mRNA:NM\_018273

Protein:NP\_060743hypothetical protein FLJ10922BL

GenBank Source:BC016919

LocusLink Home

FLJ10922 Index

Top of Page

Nomenclature

Overview

Map

RefSeq

Related Seqs

Links

LocusLink

Collaborators

Download

FAQ

Help

Statistics

RefSeq

About


Download

FAQ

Statistics

**Fig. 2.9 UniGene**

Results of UniGene search for the hypothetical protein FLJ10922, the product of the gene FLJ10922 identified on contig NT\_011109. The entry details the expression of the protein in skeletal muscle.

 NCBI

UniGene

Home Page

Query Tips

FAQ

DDD

Download UniGene

Related Resources

LocusLink

HomoloGene

dbEST

Trace Archive

BLAST

CGAP

UniGene

PubMed Nucleotide Protein Genome Structure Popset Taxonomy

Search UniGene

Limits Preview/Index History Clipboard Details

UniGene Cluster Hs.351335 *Homo sapiens*

### FLJ10922: Hypothetical protein FLJ10922

LINKS: [LocusLink](#) | [HomoloGene](#)

**SELECTED MODEL ORGANISM PROTEIN SIMILARITIES**  
organism, protein and percent identity and length of aligned region

<i>H.sapiens</i> :	<a href="#">ref.NP_060743.1</a> - 100 % / 128 aa hypothetical protein FLJ10922 (see <a href="#">ProtEST</a> ) [ <i>Homo sapiens</i> ]
<i>M.musculus</i> :	<a href="#">ref.NP_056549.1</a> - 32 % / 127 aa procollagen, type V, alpha 1; (see <a href="#">ProtEST</a> ) pro-alpha1(V) collagen [ <i>Mus musculus</i> ]

**MAPPING INFORMATION**  
Chromosome: 19  
Genome View: [Chromosome 19](#)  
UniSTS entries: [stSQ53674](#) [Map View](#)  
UniSTS entries: [RH102358](#) [Map View](#)

**EXPRESSION INFORMATION**  
cDNA sources: 2 pooled tumors (clear cell type); adenocarcinoma; brain; moderately-differentiated endometrial adenocarcinoma, 3 pooled tumors; NEUROBLASTOMA COT 25-NORMALIZED; cervical carcinoma cell line; myeloid cells, 18 pooled CML cases, BCR/ABL rearrangement positive, includes both chronic phase and myeloid blast crisis; placenta; hepatocellular carcinoma; Liver and Spleen; carcinoid; pectoral muscle (after mastectomy); kidney; duodenal adenocarcinoma, cell line; placenta\_normal; hypernephroma, cell line; melanotic melanoma, high MDR (cell line); cartilage; pooled colon, kidney, stomach; Purified pancreatic islet; human retina; fetal eyes, lens, eye anterior segment, optic nerve, retina, Retina Foveal and Macular, RPE and Choroid; RPE and Choroid; Ascites; breast; ovary, tumor tissue; Liver; myeloma; astrocytoma grade IV, cell line; pooled pancreas and spleen; human skeletal muscle; large cell carcinoma; heart\_neonatal; embryonal carcinoma, cell line; pineal gland; mixed (pool of 40 RNAs); RPE/choroid; whole brain  
SAGE: [Gene to Tag mapping](#)

### 2.5.2 MOLECULAR ANALYSIS OF CHROMOSOME 10

Clinical and genetic studies have highlighted the existence of at least two additional Miyoshi Myopathy (MM) loci, with a second MM locus being tentatively assigned to chromosome 10p (Linssen *et al.*, 1998). The chromosome 10 markers D10S547, D10S2325, D10S570 and D10S191 which were used in Linssen's study, were used for haplotype analysis of our families 7-15 in order to establish linkage to chromosome 10. Further haplotype analysis was performed using additional microsatellite markers mapping distally and proximally to the original region of interest in order to identify recombinant boundaries in any families showing linkage.

The map order and location of the markers was established by the same methods as those described in section 2.5.1 for the genetic analysis of chromosome 19. Similarly, the primer sequences and PCR product sizes were obtained as described previously in section 2.5.1.

### 2.5.3 MOLECULAR ANALYSIS OF CHROMOSOME 3

In the non-dysferlin MM families haplotype analysis was also performed for the caveolin 3 region on chromosome 3. Caveolin 3 mutations cause several types of muscular disorders, including LGMD1C, hyperCKemia, rippling muscle disease, and distal myopathy. Caveolin 3 interacts with dysferlin by co-immunoprecipitation analysis. Caveolin 3 expression has been shown to be reduced in dysferlin deficient muscle and vice versa in some caveolin 3 deficient muscle dysferlin expression is reduced. Caveolin 3 therefore appears to be a good candidate for Miyoshi Myopathy not linked to dysferlin.

Four microsatellite markers mapping within and flanking the caveolin 3 gene were analysed: D3S3611, D3S3691, D3S1307 and D3S2170.

The map order and location of the markers was established by the same methods as those described in section 2.5.1 for the genetic analysis of chromosome 19. Similarly, the primer sequences and PCR product sizes were obtained as described previously in section 2.5.1.

## **2.6 SSCP ANALYSIS**

SSCP (single-stranded conformation polymorphism) is a method for detection and localisation of uncharacterised point mutations based on the property of single stranded DNA molecules of the same length to assume different conformations depending on their nucleotide sequence. The method is a scanning one allowing only detection and localisation of mutations; further scanning followed by DNA sequencing would be necessary in order to specify the mutation. Detection of mutations responsible for particular phenotypes allows for molecular characterisation, diagnosis, prevention and treatment of inherited diseases such as Muscular Dystrophy.

Single-stranded DNA molecules fold into complex three-dimensional structures as a result of intrastrand base pairing. Changes of as little as a single base causes variation in the looping and compaction of the secondary structure, which changes electrophoretic mobility through native polyacrylamide gels. Hence single strands of equal length but different sequence can vary considerably in electrophoretic mobility. SSCP exploits the differences in mobility between wild-type and mutant strands of DNA (Orita *et al*,

1989a,b,1990; Ainsworth *et al.*, 1991; Dean and Gerrard 1991; Condie *et al.*, 1993; Glavac and Dean 1993; Fan *et al.*, 1993; Axton and Hanson 1998; Jaeckel *et al.*, 1998; Nataraj *et al.*, 1999.

SSCP involves three steps:

- 1) amplification by PCR of the target region of the gene
- 2) denaturation of the PCR product
- 3) electrophoresis of the single stranded DNA through MDE gel at neutral pH

#### 1) PCR amplification of gene exons for SSCP

Primers were identified by bioinformatic analysis using the resources LocusLink and Sequence Viewer at the NCBI (National Center for Biotechnology Information) website, whose home page is at <http://www.ncbi.nlm.nih.gov>. Exons were identified for each gene using LocusLink. These sequences were accessed using Sequence Viewer and primers then designed. Table 2.3 summarises the primers used and their sequences. DNA was amplified from genomic DNA templates under standard conditions as described for microsatellite loci in section 2.3. Care was taken to minimise the production of non-specific amplification products, as these would complicate the interpretation of results. Optimisation was carried out as described in section 2.3, but in this case it was sometimes necessary to increase the annealing temperature to 60°C.

The use of double stranded DNA as a template allowed both strands of the target region to be analysed simultaneously. This poses an advantage over the use of single stranded templates as each strand behaves differently and one will often produce better conformers for SSCP than the other.

Primer	Forward sequence 5' → 3'	Reverse sequence 5' → 3'
FXVD1EXON1	ACT GCC TAA TCC GTG GTG TC	CAA CTT GGT TCT TCG GTC CC
FXVD1EXON2	CCC TGA TTT CTC TCT CTT TCC A	AAT CAA AGG ACC AGC AAA GC
FXVD1EXON3	TGC TCT GCT GCT CAC AGA CT	AGC TAG GGG CAC TCA CTC AG
FXVD1EXON4	CTT CTC CTA CCT CTC CAC GC	GTA CTC CCC GCC TCA CCC
FXVD1EXON5	CTC TGC CTC TGT CTT CCC AG	CCC AGA ATA CCC GCA GTC TC
FXVD1EXON6	CAG GTC TGT CCA CCG CAG G	CTC CCT TCC TCC CTC CTT C
FLJ20200 EXON1A	AGT CTG GTG AAT GGA CAC	CAG GAG CCC AGC GGG ATG G
FLJ20200 EXON1B	GGT GGT GGT GGC TCC CTC TG	TCC CAC CCG AGT GAC CCC G
FLJ20200 EXON2	GAA TGG GGA CTC ACA GGA	GGT GGC CGT GGT CAT GG
FLJ20200 EXON3	TGA GGG AAA GTG GGG TAT AG	TCT GGA GGG AGC CTG AAT
FLJ20200 EXON4A	TTC TGG CAC CAG CCA CAA G	CGT CTC CCC TGC TAC CCC GGG GC
FLJ20200 EXON4B	TAG AAT GTC ACT GAG TGC CG	GAC TCT GAC CCC CTA CTC CC
FLJ20200 EXON5	AGA TTT GGC TTC GGC TCC	CCT CTC TTT CCC TCC ACA
FLJ10922 EXON1	CCG ATT TCT CCC TCA CCT TA	CTT TGC GCA TGC GTT TTA CT
FLJ10922 EXON2A	GGA CAG GCG GTA CCT GTA TT	GGT CCA GGG TCC GAG TAT G
FLJ10922 EXON 2B	AAC AGT GGC CAT ACT CGG AC	CTA GAT GGC CTT TGA CCC CT
FLJ10922 EXON3	GAA CAA GCA CCA CAG GGC	TTT GCT TCC TGA GGT AAC AGG
FLJ10922 EXON4	CTG GGG ATC CGT TAG TGA TG	CCT ATC CCA TCT TTT TCT CTC TGA
SEPW1 EXON1	CAG GTG GGA GGT TAG TGT GG	GCT GGG CTT ACC AAT AAA CG
SEPW1 EXON2&3	ACT CTC CTA CCA GTG GCG CT	AGT CTT TCT CCC CAT CCC AA
SEPW1 EXON4&5	CCG GGT TCT TTG AAG TGA TG	CCC TCA CCT CTG CCT TCA G

Table 2.3 Primers used for SSCP analysis

## 2) Denaturation

Prior to loading on the gel, double stranded DNA was denatured by incubation at 94°C for 4 min in the presence of SSCP buffer (95% formamide, 20mM EDTA, 0.05% xylene, 0.01% bromophenol blue). 5-8µl of completed PCR product was used depending upon concentration (determined by electrophoresis, see section 2.2), combined with equal volume of SSCP buffer.

## 3) Electrophoresis

Conformers of single stranded DNA were separated by gel electrophoresis under nondenaturing conditions. Optimum resolution is obtained using cross-linked polyacrylamide from the Hydrolink series of gel matrices (Molinari *et al.*, 1993) which are marketed under the trade name of MDE (Mutation Detection Enhancement), (FMC BioProducts). 50% MDE gels were used (50% Hydrolink/MDE, 6% 10X TBE, 5% glycerol, 6% Ammonium persulfate, 0.15% TEMED). Electrophoresis was carried out in 0.6X TBE buffer for 24 hours at 240V using the BIO-RAD PROTEAN® IIxi Cell. This apparatus maintained gels at a constant temperature, necessary to prevent dissociation of intrastrand base pairs, destabilisation of conformers and hence loss of resolution.

Following electrophoresis, conformers were detected by silver staining. The MDE gel was fixed by immersing in fixative (10% ethanol, 0.5% acetic acid) for 3 minutes, followed by a further 3 minutes in fresh solution. The gel was then incubated in silver nitrate (0.1% w/v) for 15 minutes. The gel was then rinsed in distilled water and incubated in an aqueous solution of 1.5% sodium hydroxide and 0.15% formaldehyde for

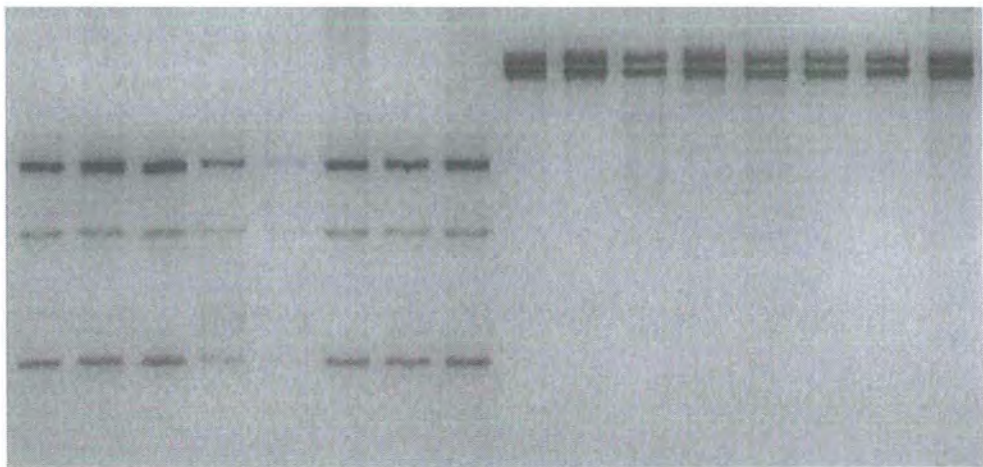


20 minutes. The stained gel was then fixed by immersion in a 0.75% aqueous solution of sodium carbonate for 10 minutes.

An example of a stained SSCP gel is shown in Fig. 2.10.

**Fig. 2.10 Single stranded conformational polymorphism (SSCP)**

Conformers of single stranded DNA separated by gel electrophoresis through an MDE Hydrolink gel under native conditions. Samples produced by amplification of exons 2A and 2B of the gene FLJ20200 in Family 2. All samples are normal with no evidence of mutation, since conformers are identical between individuals.



## **CHAPTER 3**

### **RESULTS**

### **3.1 HAPLOTYPE ANALYSIS OF THE LGMD2I REGION**

Six potential LGMD2I families were analysed using the following microsatellite markers mapping to the LGMD2I region: D19S606, D19S596, D19S902 and D19S879. Haplotype analysis had previously excluded the families from the limb-girdle muscular dystrophy loci LGMD2A, LGMD2B, LGMD2C, LGMD2D, LGMD2E and LGMD2F. No chromosome 19 data was available for the families with the exception of Family 1. In this family haplotype analysis had been performed using the following markers: D19S918, D19S412, D19S606, D19S879, D19S867. The haplotype data assembled for Family 1 is shown in Fig. 3.1. The data is suggestive of linkage, with both affected individuals showing shared alleles for five of the markers studied, but the data is uninformative.

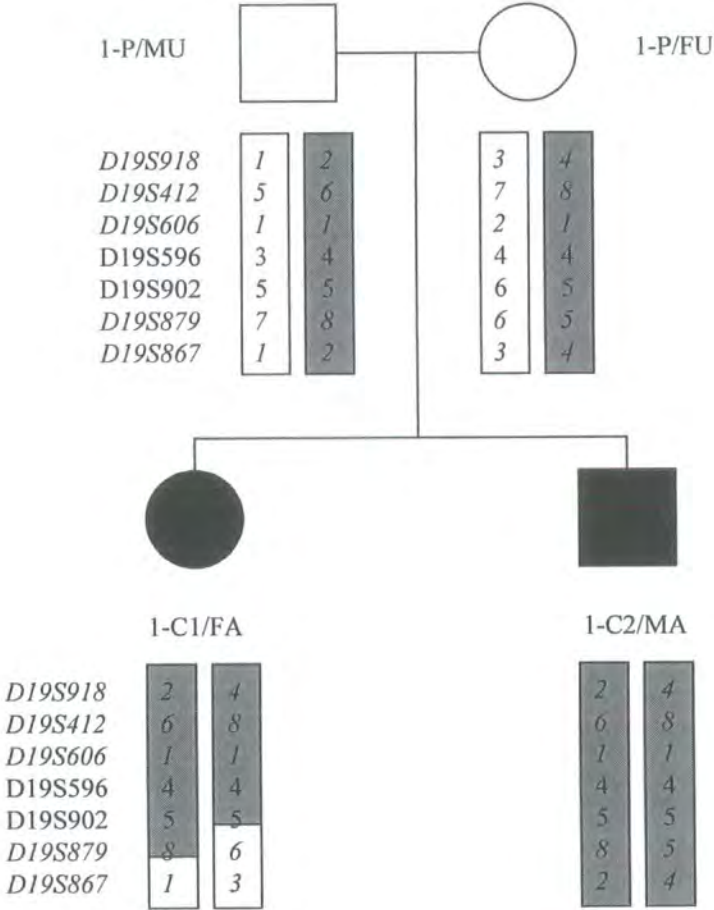
In Family 2 marker D19S606 is uninformative, and the markers D19S596, D19S902 and D19S879 identify recombinants by comparing the haplotypes of the two affecteds. Both affecteds show different haplotypes, suggesting exclusion from the LGMD2I locus (Fig. 3.2).

In Family 3 the data is consistent with linkage (Fig. 3.3). Both affected individuals share the same haplotypes, which are not shared by the two unaffected sibs. There is no evidence of recombination among the affected individuals.

In Family 4 the two affecteds do not share the same haplotypes for markers to the LGMD2I region. Haplotypes are however shared by the affected individual 4-C3/FA and the unaffected sib 4-C1/MU. Similarly the affected individual 4-C8/FA shares the same

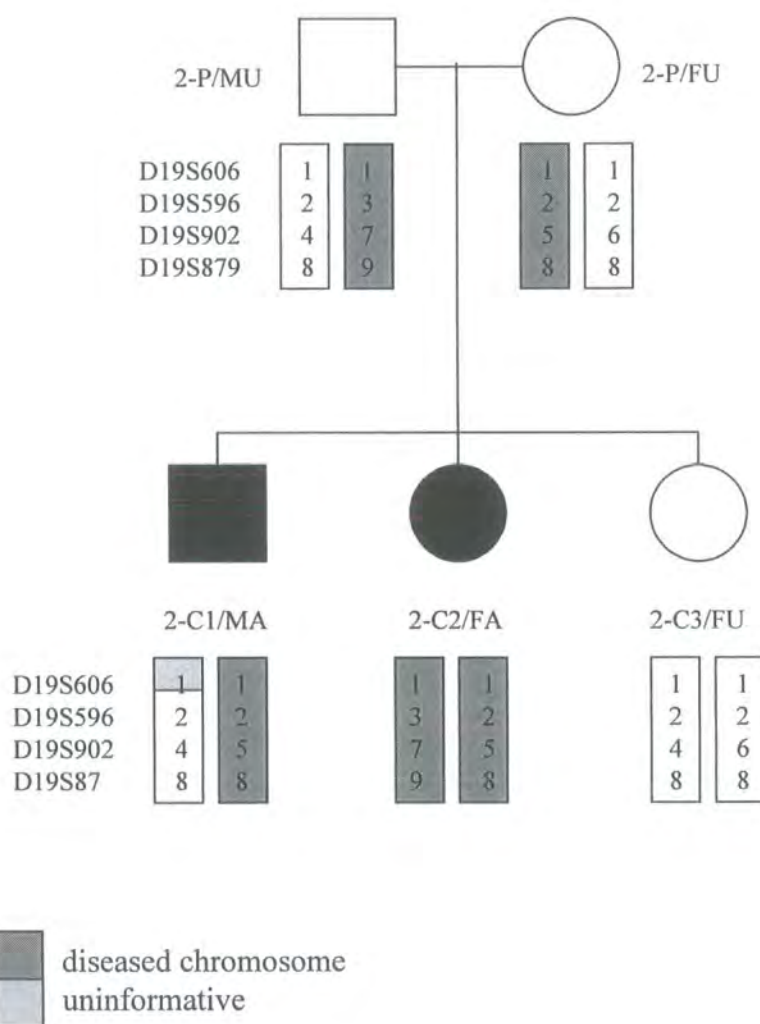
**Fig. 3.1 Chromosome 19q haplotyping in Family 1**

Data in italics generated by Angela Huebner's Group in Germany. Order of markers was determined from sequence data for the region and is given centromere → telomere (top → bottom). Affected individuals are represented by solid symbols and unaffected individuals by open symbols.



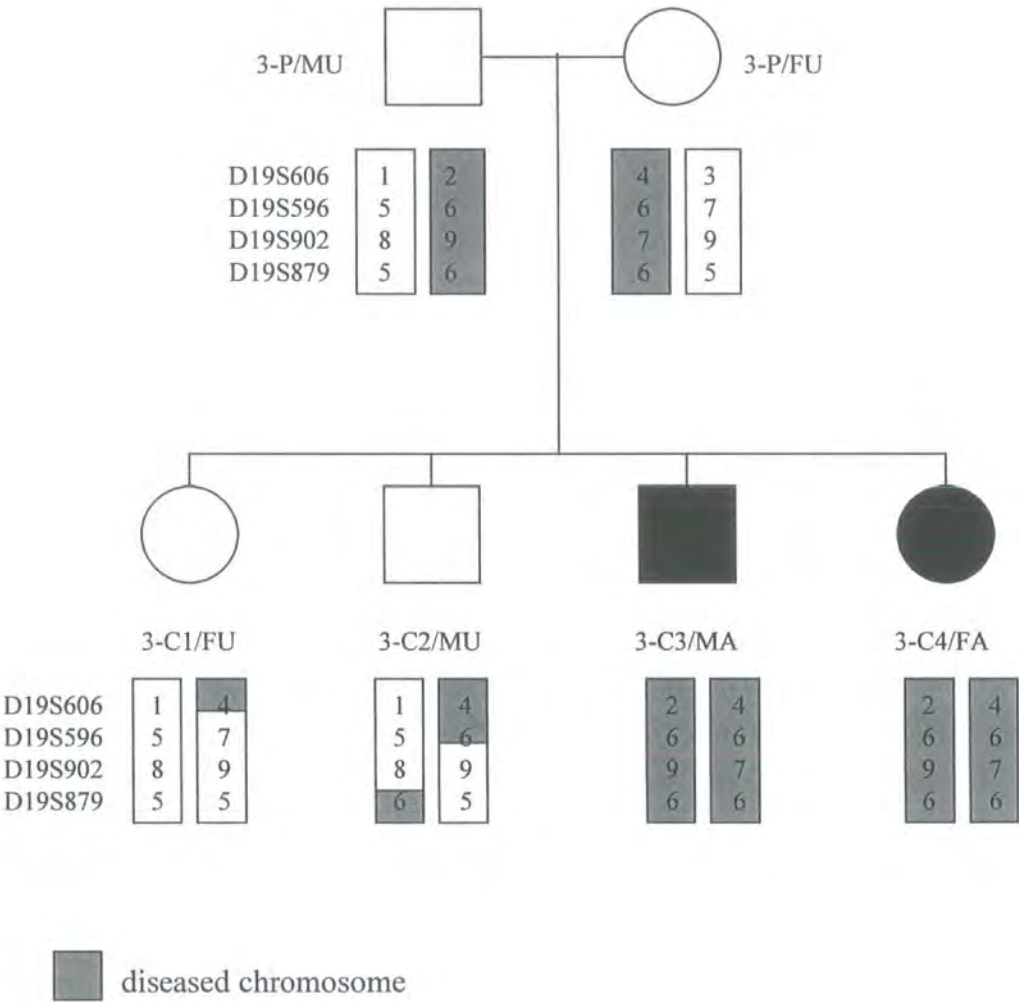
**Fig. 3.2 Chromosome 19q haplotyping in Family 2**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given centromere → telomere (top → bottom).



**Fig 3.3 Chromosome 19q haplotyping in Family 3**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given centromere → telomere (top → bottom).



haplotype as the unaffected 4-C5/MU. This data is consistent with exclusion from the LGMD2I locus (Fig. 3.4).

In Family 5 the two affecteds share the same haplotype, which is not seen in the unaffected sibs. The marker D19S606 is uninformative. For the other markers there is no evidence of recombination. The data suggests linkage to the LGMD2I locus (Fig. 3.5).

In Family 6 the two affected individuals share the same haplotype. However the markers D19S606 and D19S596 are uninformative, and there is no evidence of recombination. The data suggests linkage to the LGMD2I locus, but is not fully informative as there are no unaffected sibs in the family (Fig. 3.6).

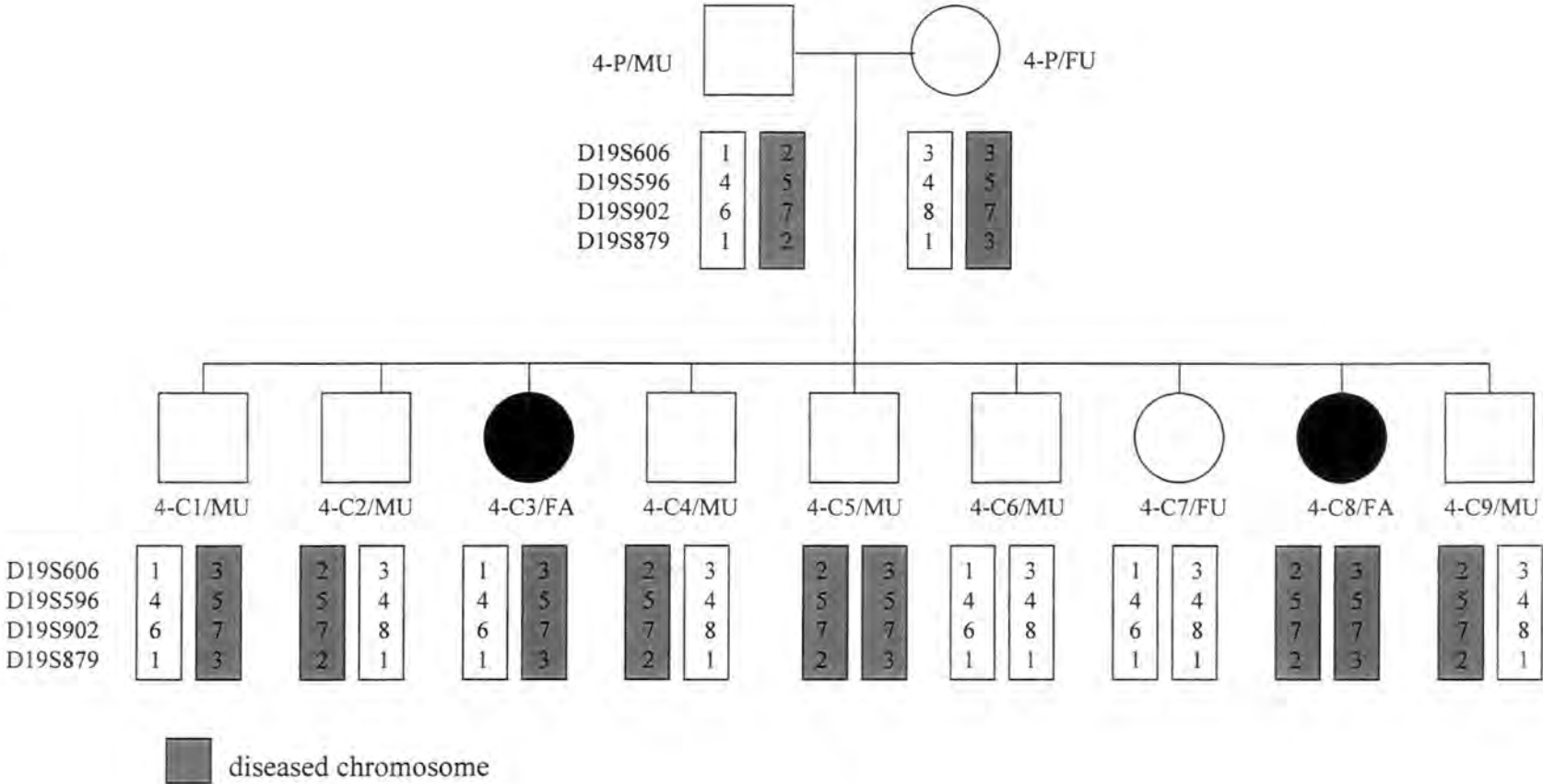
### **3.2 PHYSICAL MAPPING OF THE LGMD2I REGION**

Identification of ESTs mapping to the LGMD2I critical region between the centromeric marker D19S606 and the telomeric marker D19S879 was performed by computer based analysis using information from the GeneMap 1998. Having now been fully sequenced, the GeneMap for this region is no longer available. The ESTs identified are given in Table 3.1. The ESTs were subjected to BLAST (Basic Local Alignment Search Tool) analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) with the HTGS (High Throughput Genomic Sequencing) database in order to identify genomic sequence contigs. It was possible to identify overlaps where several ESTs and microsatellite markers were assigned to the same contig. From these analyses a primary physical map of the LGMD2I region was constructed (see Fig. 3.22).



Fig. 3.4 Chromosome 19q haplotyping in Family 4

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given centromere → telomere (top → bottom).



**Fig. 3.5 Chromosome 19q haplotyping in Family 5**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given centromere → telomere (top → bottom).

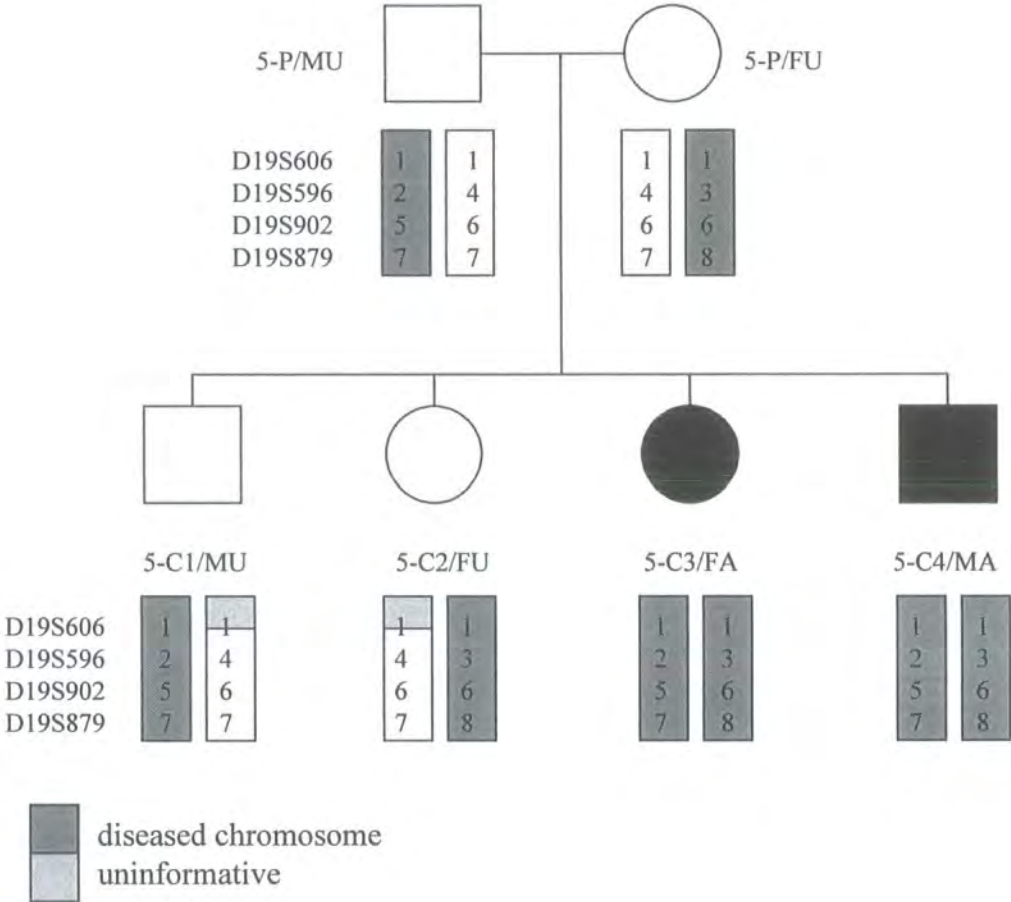


Fig. 3.6 Chromosome 19q haplotyping in Family 6

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given centromere → telomere (top → bottom).

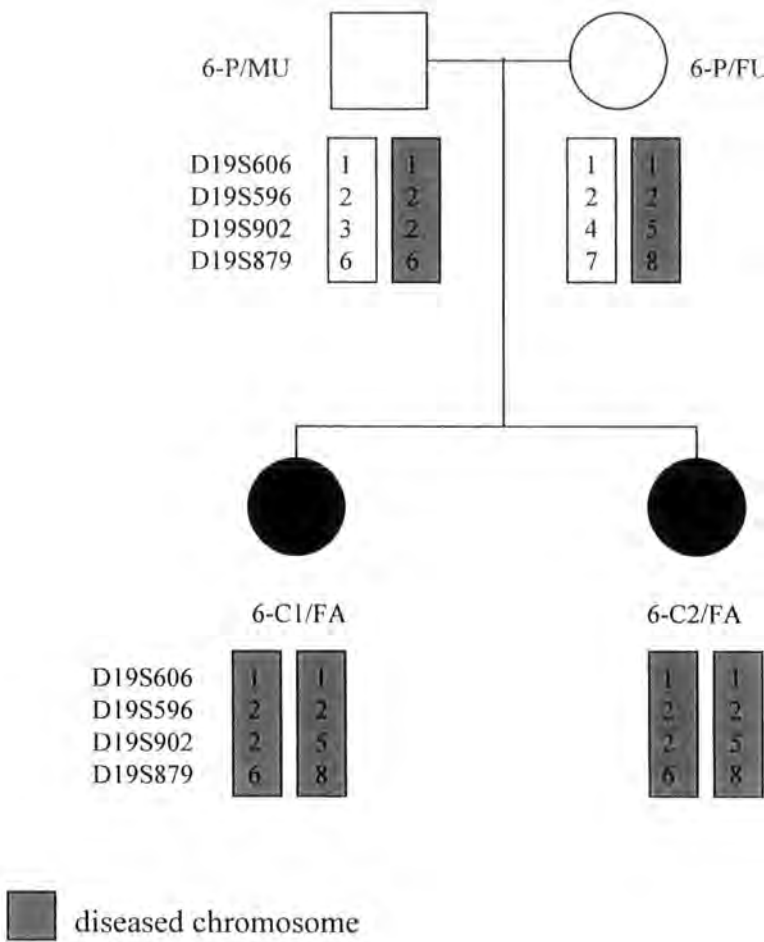


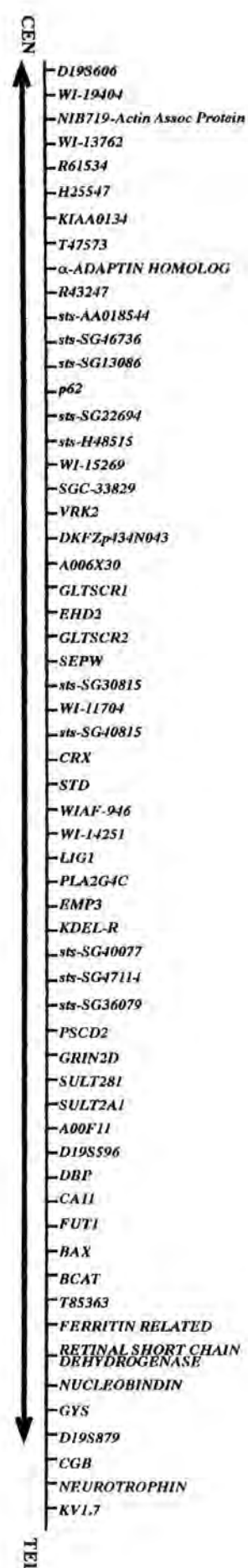
Table 3.1 ESTs mapping to the LGMD2I critical region

EST	GENE		CONTIG CLONE
WI-9140	GRLF1	glucocorticoid receptor	ACO12313
SGC36947		ESTs GTSCR2	
stSG44765		ESTs also expressed in muscle	
stSG53674		ESTs	ACO73131
WI-19271		Human mRNA	
H56372		ESTs	
stSG49271		ESTs	
RP_L18	RPL18	Ribosomal protein L18	
Cda13a10		ESTs	
R99562	HNF3G	hepatocyte nuclear factor 3	
stSG3117	ATP5G1	ATP synthase, H+ transporting	ACO06262
sts-AA027108		ESTs	
AFM284yg5	D19S412	Microsatellite marker	ACO07193
A006X30		Homo sapiens mRNA	ACO10326
stSG2167		ESTs	ACO08635
stSG52313		ESTs	ACO24740, 23094
stSG12792		EST	
AFMa133zh9	D19S596	Microsatellite marker	ACO24740, 09002, 23094
NIB719		Homo sapiens actin associated protein	ACO16589, 10331, 73548
stSG47680		ESTs	ACO08755, 24443
stSG1238	SULT2A1	sulfotransferase family 2A	ACO08745, 24582, 11457, 40922
stSG44984		ESTs	
sts-T47573		ESTs	ACO08754, 08576
stSG465	NAPA	N-ethylmaleimide-sensitive factor	
sts-U13061		Unknown	
sts-T77818		ESTs	
stSG35241		EST	
H48539		EST	ACO08895, 27709
stSG3439		ESTs	
stSG52618		ESTs	ACO08532
WI-22443		ESTs	ACO08532
H25547		ESTs	ACO08754
stSG30190		Homo sapiens mRNA	ACO08755, 24443
stSG8858		ESTs	ACO08755, 24443
stSG58588		ESTs	
sts-N25366		ESTs	ACO0875
sts-F16347		Unknown	
EST196348		ESTs	ACO08888
stSG4486	NAPA	N-ethylmaleimide-sensitive factor	
WI-13762		ESTs	
WI-20785	SEPW1	selenoprotein W, 1	
AA057647		ESTs	ACO08755
U82987		Human Bcl-2 binding component 3	ACO08532, 17110
Cda11g02	PLA2G4C	phospholipase A2, group IVC	
sts-R61534		ESTs	
stSG26816	DBP	D site of albumin promoter	ACO40922, 08888
WI-14200	PLA2G4C	phospholipase A2, group IVC	ACO10458, 11466
EST	GENE		CONTIG CLONE
sts-M35531	FUT 1	fucosyltransferase 1	

stSG47114		ESTs	ACO08403, 73131
D66904	HRMT1L2	HMT1	
stSG2506		ESTs	
A004H25	PLA2G4C	phospholipase A2, group IVC	
stSG12712	EMP3	epithelial membrane protein 3	ACO73131, 08392
stSG30258		ESTs	
WI-9028		Human mRNA	ACO73131
WIAF-946		Homo sapiens clone IMAGE	
sts-U59752	PSCD2	pleckstrin homology	ACO73131
stSG39919	GRLF1	glucocorticoid receptor	
stSG40815		ESTs	ACO08745, 24582, 11457
stSG9290		Homo sapiens clone IMAGE	ACO11466, 11212
stSG30815		EST	ACO08745
WI-14251		Homo sapiens mRNA	ACO11466, 11212
WI-11704		ESTs	ACO08745, 21988, 11481, 26635
stSG36079		EST	ACO08403
WI-16013		ESTs	ACO40922
A004F11		ESTs	
stSG42757	FUT1	fucosyltransferase 1	ACO08392
stSG54061		ESTs	ACO26803, 08749
stSG63102		ESTs	ACO08392
stSG40077		ESTs	ACO08403
stSG54041		Homo sapiens orphan neurotransmitter	ACO08403
stSG55012	CA11	carbonic anhydrase XI	ACO68888, 40922, 23094
stSG27366		Homo sapiens RUVBL2 protein	ACO08687
WI-19404		EST	ACO73548, 16589, 10331
stSG39537		H. sapiens apoptosis associated protein	ACO26803, 24749, 08749
stSG15451		Homo sapiens clone DT1P1A2 mRNA	ACO26803, 08749
stSG44262		ESTs, retinal short chain dehydrogenase	ACO26803, 24740, 08749
sts-R32980		Homo sapiens apoptosis associated	
stsSG8002		ESTs	ACO26803, 24740, 08749
A002E14		Homo sapiens RUVBL2 protein	
sts-W47119		ESTs	
A002I45		ESTs	
stSG39666	HRC	histidine-rich calcium-binding protein	ACO08891
sts-AA036925		EST, similar to ferritin light chain	ACO26803, 08749
sts-T85363		ESTs	ACO26803, 24740, 08749
sts-Z41308		Homo sapiens actin associated protein	ACO73548, 16589, 10331
WI-21237	BCAT2	branched chain aminotransferase 2	ACO26803, 24740, 8749
WI-15464		Human clone 23867 mRNA sequence	
stSG26844	BAX	BCL2-associated X protein	ACO08749, 26803
WI-15345	CD37	CD37 antigen	
A005S30	PSCD2	pleckstrin homology	ACO73131
stSG1742		ESTs	
stSG39641	CGB	chorionic gonadotropin	ACO08687
sts-R49148		ESTs, weakly similar to VRK2	ACO14527, 211543
stSG62383		ESTs	
stSG96	SNRP70	small nuclear ribonucleoprotein	
EST	GENE		CONTIG CLONE
sts-J00117	CGB	chorionic gonadotropin	
stSG30581		EST, from retina	ACO08687
stSG15187		ESTs	

SGC30695		ESTs	
SGC34723		ESTs	ACO08891
stSG30347		EST	ACO11501
SGC34294		EST	
SGC33829		ESTs	ACO21154,10624,11452
WI-15269		ESTs	ACO11452,21154
WI-12840		ESTs	
sts-H48515		ESTs, activating transcription factor	ACO11452, 21154
stSG42234		ESTs	ACO11495
stSG53476		ESTs	ACO21163
stSG44400	HRMT1L2	HMT1	
stSG22694		ESTs	ACO21163, 11452, 21163
stSG49525		ESTs	ACO08655, 19157,73646
b05503rbk		Homo sapiens clone DT1P1A2 mRNA	
stSG46478		ESTs	
stSG48278		ESTs	
sts-AA024912	CD37	CD37 antigen	
sts-AA031950		ESTs	
sts-M86528		ESTs, neurotrophin 4/5	ACO08687
Cda0se05		ESTs, hypothetical protein	
stSG25992		ESTs, neurotrohin	
stSG48023		ESTs, similar to C. elegans	
sts-U03858	FLT3LG	fms-related tyrosine kinase 3	
stSG52049		ESTs	ACO21154, 10624, 11452
sts-T97512		ESTs, weakly similar to zinc finger protein	
Cda19e04		Homo sapiens mRNA	ACO11452,21154
stSG53174		ESTs	ACO08743
stSG31958		EST	ACO08743
stSG9592		ESTs	ACO21163
sts-N90422	HRMT1L2	HMT1	
stSG58521		Homo sapiens CGI-25 protein mRNA	ACO11495
R43247		ESTs	ACO21163
sts-H72005		ESTs	ACO11495
Bdya0h04		ESTs, similar to mouse myosin	ACO8655, 19157
sts-T86699		ESTs	ACO8655, 19157
AFMb059wg9	D19S907	Microsatellite marker	
sts-AA018544		ESTs	ACO21163
IB1639		Human orphan receptor mRNA	ACO73646,08655
stSG49939	POLD1	polymerase (DNA directed)	ACO08655, 20909,73646
stSG54844		ESTs	ACO11495
stSG41482		EST	ACO11495
stSG38929		Human mRNA for p62	ACO11452/21163
SGC33962		ESTs	
stSG42123		ESTs	ACO11495
stSG46736		ESTs, polynucleotide kinase phosphatase	ACO21163
stSG13086		EST	ACO21163
EST	GENE		CONTIG CLONE
stSG10339		ESTs	
sts-W95460		ESTs	
stSG35523		ESTs	
stSG27382	POLD1	polymerase (DNA directed)	
stSG63150		EST, found only in testes	

stSG51006		ESTs	ACO73646
stSG42415		ESTs	
stSG47313		ESTs	ACO20909, 73646
NIB1805	IRF3	interferon regulatory factor 3	ACO11495
SGC34742	IRF3	EST	
stSG32045		ESTs	ACO73646



### LGMD2I CRITICAL REGION

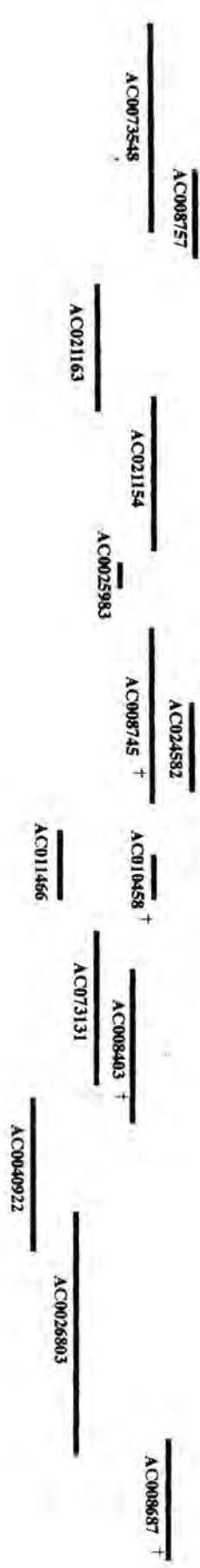


Fig. 3.22 Primary physical map of the LGMD2I critical region on chromosome 19q13.3.

The arrowed bar indicates the LGMD2I critical region. BACs highlighted with the symbol have been fully sequenced.



Following identification of the contigs, we began analysing any known and novel genes mapping to the contig using LocusLink (<http://www.ncbi.nlm.nih.gov/LocusLink/>). Using this resource a gene profile of expression, gene structure, domains, homologies and function where known to be was built for each novel transcript, with the aim of generating a contiguous transcript map of the LGMD2I critical region.

However, during this time the LGMD2I gene was identified by Francesco Muntoni's group in London. Mapping to an identical region on chromosome 19q13.3 as the severe congenital muscular dystrophy MDC1C, Muntoni demonstrated that LGMD2I and MDC1C are allelic disorders, caused by mutation in the fukutin-related protein gene (FKRP) (Brockington *et al.*, 2001). The gene maps to a region on chromosome 19q between the markers D19S219 and D19S606 (centromere-D19S219-FKRP-D19S606-telomere), a region spanning 3cM. This publication is given in the Appendix.

The focus of this study then shifted to the identification of FKRP linked families from our potential LGMD2I families (families 1-6).

### **3.3 FKRP MUTATIONS**

A collaboration was entered into with Francesco Muntoni of the Dubowitz Neuromuscular Centre, Department of Pediatrics, Faculty of Medicine, Imperial College, Hammersmith Hospital Campus, London, UK. A panel of DNA comprising affected individuals from families 1-6 was screened for mutation at London, along with an unaffected individual from each family.

Our haplotype analysis of families 1-6 identified four families which were consistent with linkage to the LGMD2I locus. Mutations in the fukutin-related protein gene (FKRP) were subsequently identified in three of the families, two of the three having a C826A mutation. Family 3 was homozygous for this change, and Family 5 was a compound heterozygote, the second allelic mutation being identified as C934T. In Family 6 a C427A mutation was identified, the second allelic mutation remaining unidentified. These findings are summarised in Table 3.2.

Table 3.2 Summary of FKRP mutations in LGMD2I

FAMILY	CHANGE	PROTEIN EFFECT	MUTATIONAL STATUS
3	C826A	Leu276Ile	Homozygous
5	C826A C934T	Leu276Ile Arg312Cys	Compound heterozygote
6	C427A	Arg143Ser  Other mutation not known	Compound heterozygote

Analysis of 17 families by Francesco Muntoni’s group resulted in the identification of a C826A mutation in 15 of the 17 families, suggesting that this is a common mutation in LGMD2I. As the C826A mutation was identified in a large number of the families studied, the possibility of this being a polymorphism was ruled out by restriction enzyme analysis, on the basis of the loss of a *Bfa*I restriction site induced by the change. On screening a panel of 200 control chromosomes only one individual heterozygous for the C826A mutation was identified, and in at least 100 of the control chromosomes the

mutation was not present. On the basis of these observations, and the fact that the mutations segregated with the disease in an autosomal recessive fashion, the mutations were considered to be pathogenic.

### **3.4 GENOTYPE-PHENOTYPE CORRELATIONS**

FKRP mutations were identified in families 3, 5 and 6 during the study at London. In family 3 a homozygous C826A mutation was identified. The two affected individuals in this family were symptomatic from age 10 years, and currently remain ambulant during the fourth decade of life. Affected individual 3-C3/MA showed markedly elevated serum creatine kinase (4105), with impairment of the proximal limb muscles, predominantly the lower limb. Cardiac abnormality was also apparent.

In family 5, a C826A mutation was again identified, along with a C934T mutation. This family displayed a more severe clinical course than that of Family 3, which was homozygous for the C826A mutation. The two affected individuals in Family 5, 5-C3/FA and 5-C4/MA (see Fig. 3.5), displayed onset of symptoms at age 9 and 7 respectively, with loss of ambulation at age 20 and 12 respectively. Both developed contractures on all leg articulations in late childhood, with trunk muscle involvement and the development of scoliosis. Progression of the disease was less severe in individual 5-C3/FA until the fourth decade, when both showed involvement of distal muscles of arms and legs, facial muscles, and hypertrophy of the tongue. Predominantly proximal atrophy was a feature, but was also apparent distally. There was also severe involvement of the respiratory muscles. Serum creatine kinase was elevated in both patients during

childhood, with individual 5-C4/MA showing normalisation during the third decade of life.

In family 6 a C427A mutation was identified, with the second allelic mutation remaining unidentified, suggesting that mutations also exist outside the FKRP coding region. Age of onset was 9 years for individual 6-C2/FA, with serum creatine kinase elevated to above 1000. The patient presented with difficulty climbing stairs, with 4 to 5 grade weakness. Significant weakness was noted in the tibialis anterior. No muscle hypertrophy was evident and the patient remains ambulant during the second decade of life. The disease in this family shows a less severe clinical course than that of Family 5, whose FKRP mutation was identified as compound heterozygote for the C826A change.

A summary of the clinical features of these LGMD2I patients is given in table 3.3.

The range of phenotypic severity due to FKRP mutations is large, and so far not observed in other forms of muscular dystrophy. 15 of the families studied at London had an identical C826A missense mutation in the FKRP gene. Five patients homozygous for this mutation had a relatively mild phenotype, whereas compound heterozygotes had on the whole a more severe phenotype, as reflected in the clinical features of families 3 and 5.

Although the patients studied at London had a variable phenotype, all were less severe than MDC1C where patients never acquired independent ambulation. It has been demonstrated that both clinically and genetically, LGMD and CMD can overlap. Approximately 40% of cases of CMD are caused by mutations in the LAMA2 gene on chromosome 6q22-23, encoding the laminin  $\alpha$ 2 chain of merosin (Tome *et al.*, 1994, Helbling-Leclerc *et al.*, 1995, Pegoraro *et al.*, 1998). Children having mutations in the LAMA2 gene usually have a severe form of CMD, MDC1A. However, a large Turkish

FAMILY	SEX	AGE (YEARS)	AGE ONSET (YEARS)	FIRST SYMPTOM	SERUM CK	MUSCLE HYPERTROPHY	WEAKEST MUSCLE	CARDIOMYOPATHY	WHEELCHAIR BOUND	MUTATION	MUTATIONAL STATUS
3	M	34	10	Toe walking, no running	4105	No	Hip girdle	Moderate LV, hypokinesia	No	C826A	Homozygous
	F	22	10	No running	ND	No	Hip girdle	ND	No	C826A	Homozygous
5	M	39	7	Waddle, arm weakness	1150	Tongue	Shoulder girdle	No	20	C826A C934T	Compound heterozygote
	F	42	9	ND	1115	Tongue	Shoulder girdle	No	ND	C826A C934T	Compound heterozygote
6	F	18	9	Stairs	>1000	No	Tibialis anterior	No	No	C427A, other mutation not known	Compound heterozygote
	M	ND	ND	ND	ND	ND	ND	ND	ND	C427A, other mutation not known	Compound heterozygote

ND = NO DATA, LV = LEFT VENTRICULAR

Table 3.3 Summary of the clinical features of LGMD2I patients

kindred having a clear LGMD phenotype, has been shown to be linked to the LAMA2 locus (Tan *et al.*, 1997). Mutations in this gene can result in either a severe disease (MDC1A) or a mild LGMD-like disease, depending on the type and location of the mutation within the gene.

A number of CMD forms have also been described which are not due to mutations in the LAMA2 gene, including Fukuyama CMD (FCMD). The FCMD gene encodes fukutin, a protein of unknown function (Kobayashi *et al.*, 1998). The fukutin-related protein gene, FKR, along with fukutin, demonstrate sequence similarities to a family of proteins involved in modifying cell surface molecules (Aravind & Koonin, 1999). The FKR gene is also mutated in a severe form of CMD, MDC1C (Brockington *et al.*, 2001), and maps to an identical region on chromosome 19q as LGMD2I (Breton & Imberty, 1999).

Mutations in the FKR gene account for a significant proportion of the patients in the London study whose severity ranged from a severe congenital form (MDC1C) to a much milder form (LGMD2I), most patients with a relatively mild disease course having the C826A mutation.

### **3.5 GENETIC ANALYSIS OF MIYOSHI MYOPATHY NOT LINKED TO DYSFERLIN**

#### **3.5.1 CHROMOSOME 10 HAPLOTYPE ANALYSIS**

In this study the genetics of Miyoshi Miyopathy (MM) not linked to dysferlin was also studied. 9 of our families showed exclusion from dysferlin on the basis of haplotyping or from the normal dysferlin expression observed in patient biopsies.

In two families (7 and 8) genetic analysis data suggested tentative linkage to the chromosome 10 locus (LOD score  $Z_{\max}(\text{D10S2325}) 2-63 \theta = 0$ ). To confirm linkage to chromosome 10 and to define the MMD2 region, further haplotype analysis was performed using the following additional microsatellite markers mapping distally to D10S189 (-D10S1713, D10S591, D10S1745-telomere), and proximal to D10S1423 (-D10S553, D10S1734, D10S1749, D10S1733, D10S1674, D10S1768-centromere). In family 7, recombinants were observed in both the affected individuals at the marker D10S1713, defining the distal recombinant boundary. The proximal recombinant boundary was not defined in this family since the markers D10S191, D10S1423, D10S553, D10S1734, D10S1749, D10S1733, D10S1674 and D10S1768 are uninformative (Fig. 3.7). In family 8, the distal recombinant boundary is defined by the marker D10S1713 in individual 8-C2/FU, confirming the observations made in family 7. The proximal recombination boundary is represented by the marker D10S1674 in affected individual 8-C6/FA (Fig. 3.8). In physical terms this represents a 25Mb region of non-recombination.

Data in *italics* generated in Holland (Linssen *et al.*, 1998). Order of markers is given telomere → centromere (top → bottom). Affected individuals are represented by solid symbols and unaffected individuals by open symbols.

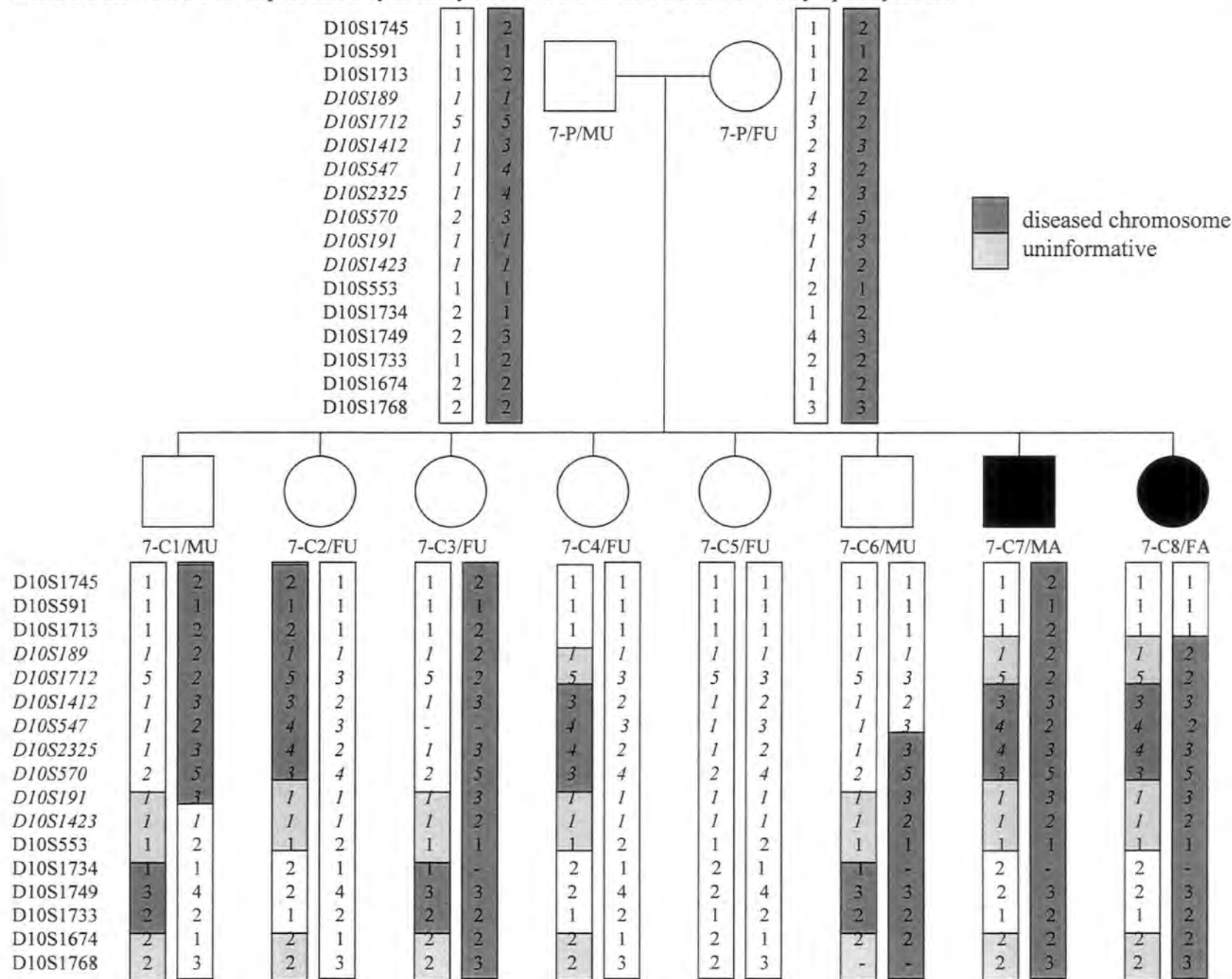
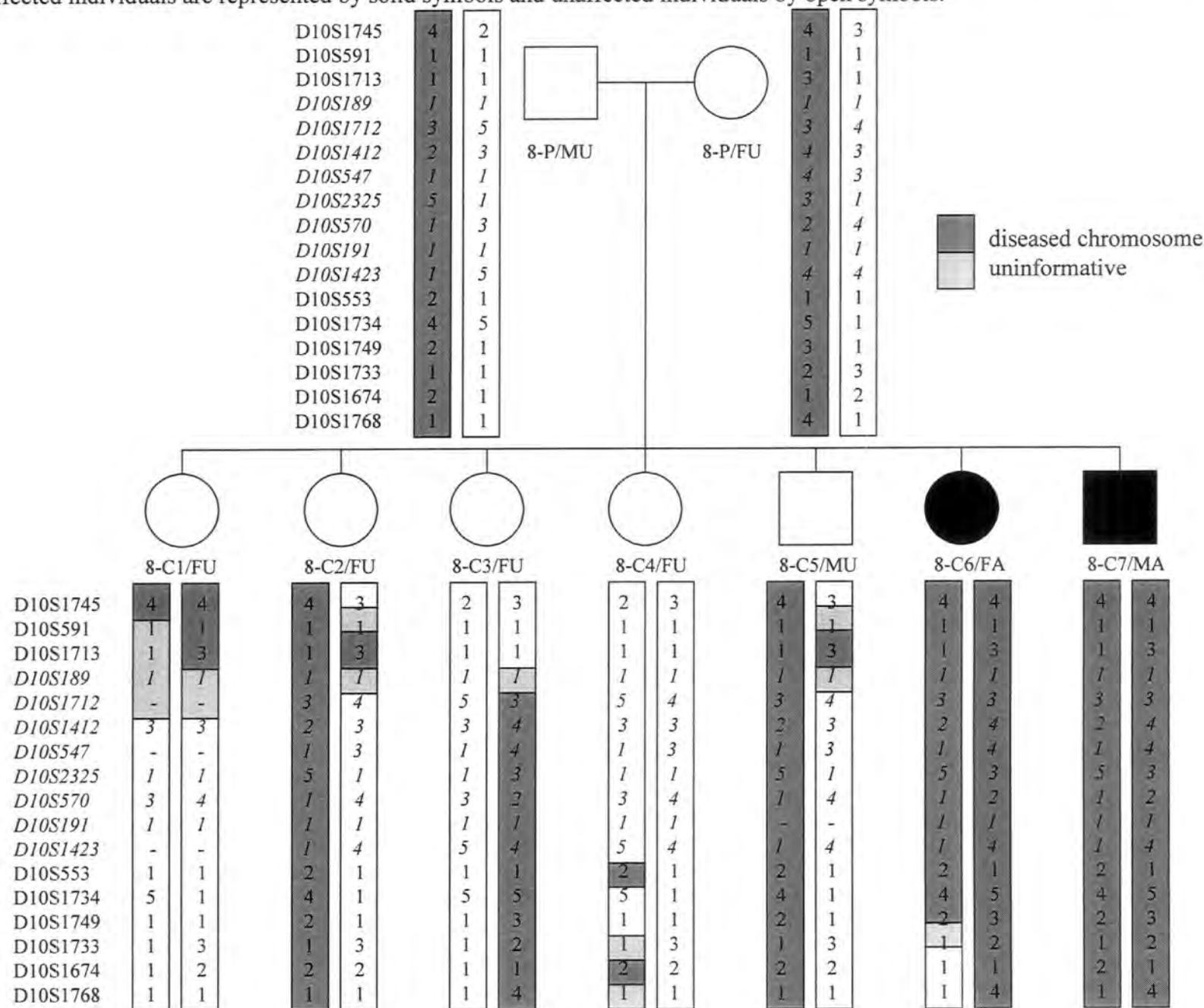




Fig. 5.5 Chromosome top haplotyping in Family 8

Data in *italics* generated in Holland (Linssen *et al.*, 1998). Order of markers is given telomere → centromere (top → bottom). Affected individuals are represented by solid symbols and unaffected individuals by open symbols.



In families 9-15 the markers D10S547, D10S2325, D10S570 and D10S191 were analysed in order to identify further chromosome 10 linked families.

In Family 9, the affected individual shares the same haplotype as the unaffected sib, suggesting that this family is excluded from the MMD2 locus (Fig. 3.9).

In Family 10 the data appears to suggest linkage to chromosome 10. The affected individual does not share haplotype with either of the unaffected sibs in the family. With further haplotype analysis the data remains consistent with linkage, but all markers were non-recombinant across a large physical region (25Mb). The proximally located markers D10S553 and D10S1674 are uninformative (Fig. 3.10). If the data from this family is evaluated with the data produced for families 7 and 8 using the GENEHUNTER program and two point linkage analysis performed, a possible LOD score of 2.83 ( $\theta = 0$ ) at the marker D10S2325 can be generated.

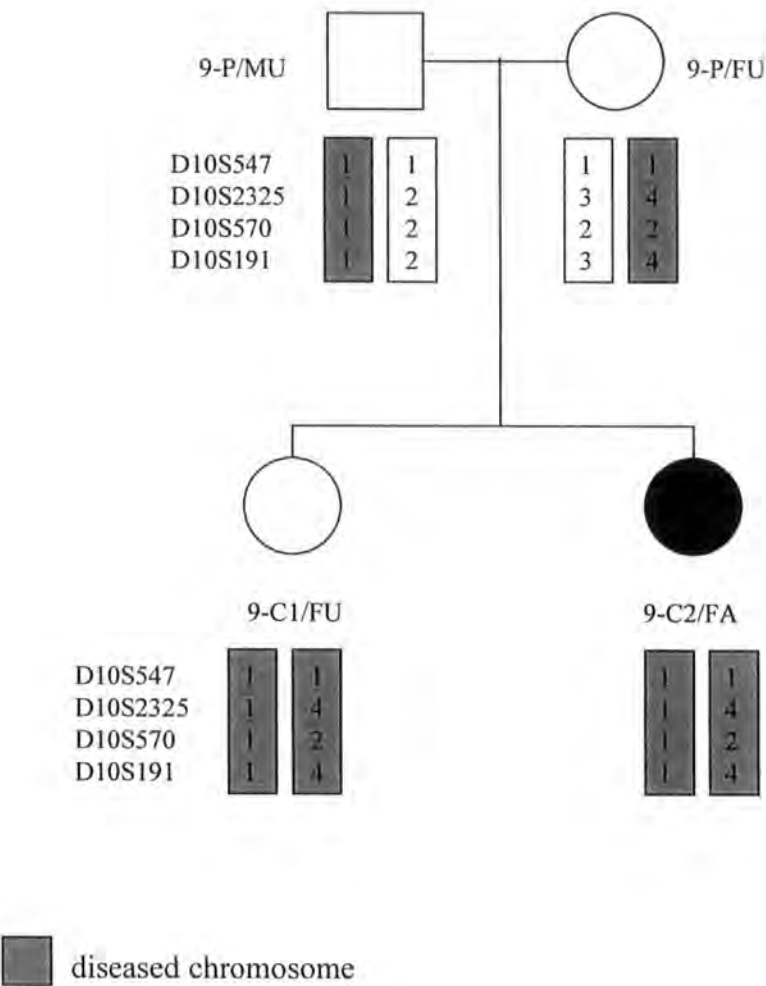
In Family 11, recombination is seen in both affected individuals at the marker D10S191, but the two affecteds do not share the same haplotype. Exclusion cannot be confirmed, and requires analysis of additional markers as in family 10 (Fig. 3.11).

In Family 12, the affected individual does not share haplotype with either of the two unaffected sibs, and there is no evidence of recombination (Fig. 3.12). Again, to obtain informative results further markers need to be analysed in order to establish linkage.

In Family 13 the data identifies recombination at the marker D10S191 in individual 13-C3/FU. The two affected individuals share the same haplotype generated from markers D10S547, D10S2325, D10S570 and D10S191, but the unaffected individual 13-C4/FU also shares this haplotype (Fig 3.13). This suggests no linkage to chromosome 10, but to confirm it is necessary to analyse more markers as in family 10.

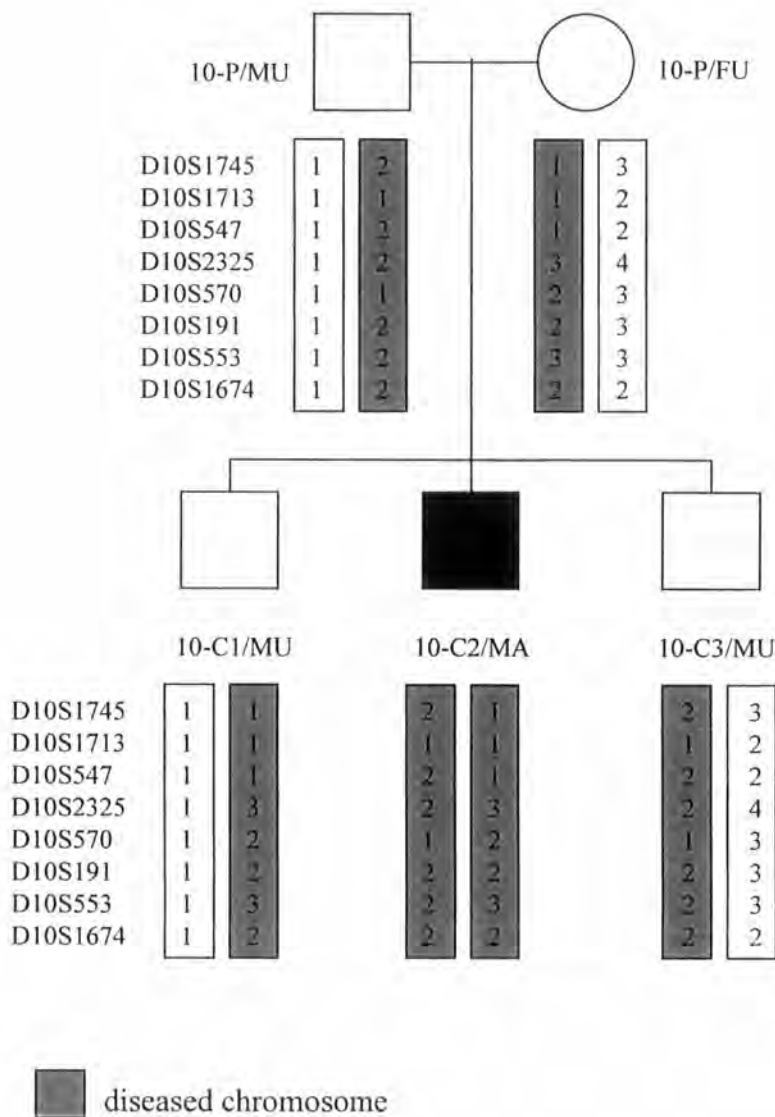
**Fig. 3.9 Chromosome 10p haplotyping in Family 9**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



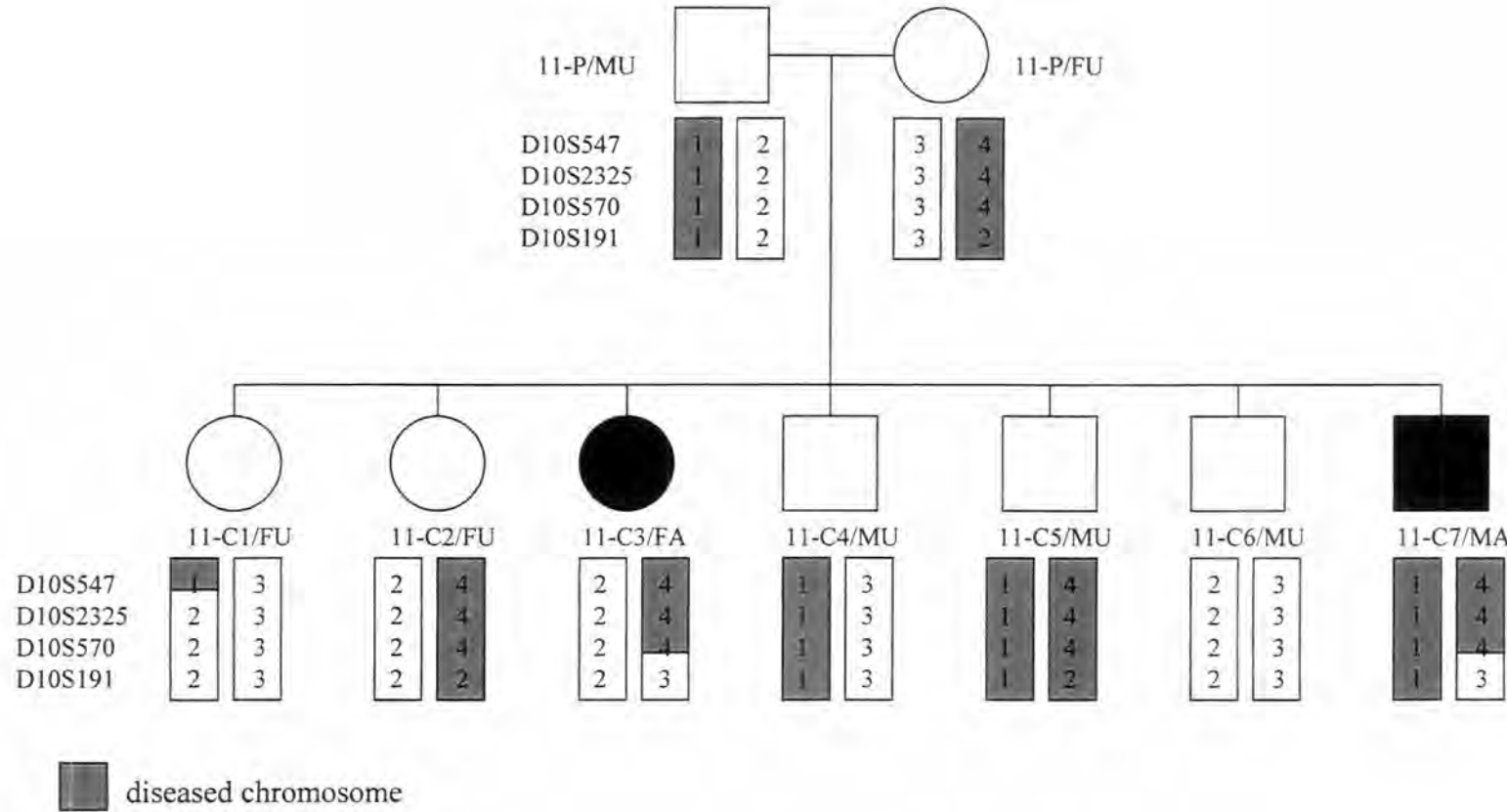
**Fig. 3.10 Chromosome 10p haplotyping in Family 10**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



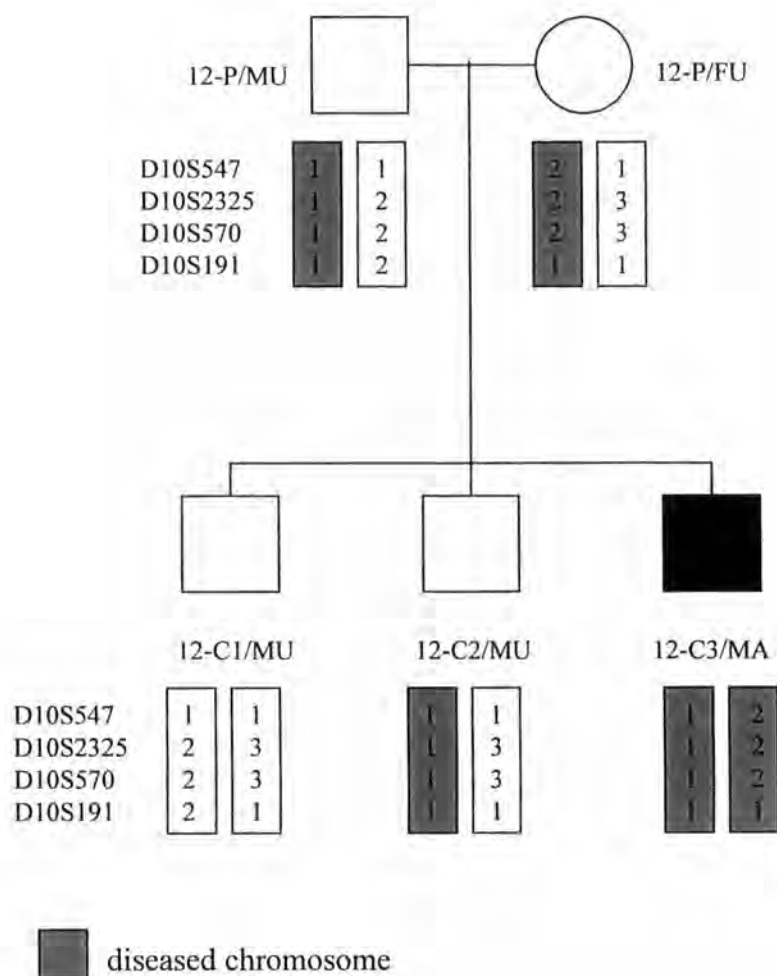
**Fig. 3.11 Chromosome 10p haplotyping in Family 11**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



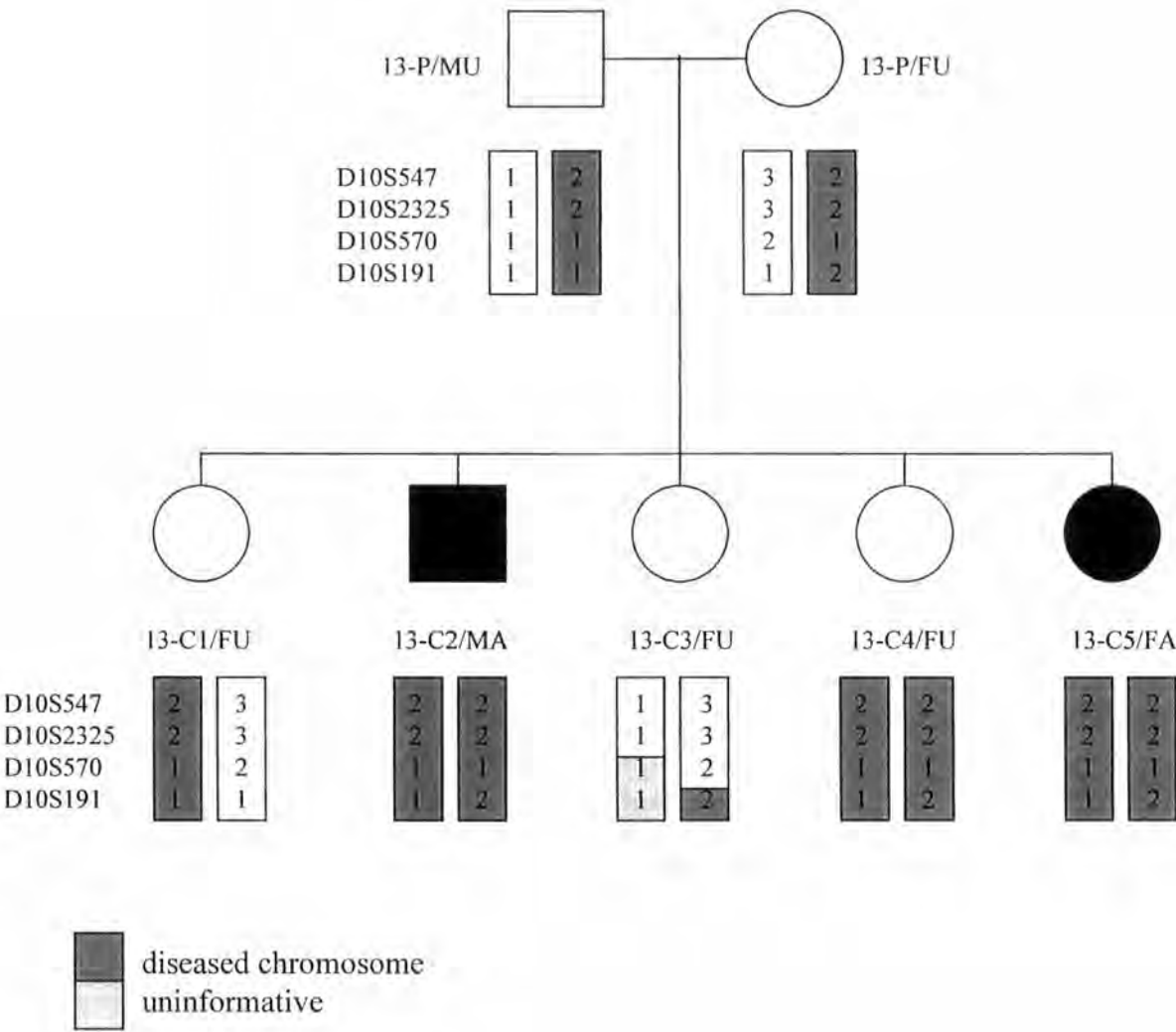
**Fig. 3.12 Chromosome 10p haplotyping in Family 12**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



**Fig 3.13 Chromosome 10p haplotyping in Family 13**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



Similarly, for Family 14 the affected individual shares the same haplotype as the unaffected sib 14-C1/FU (Fig. 3.14), and further markers would need to be analysed in order to confirm exclusion from chromosome 10.

In Family 15, there are no shared haplotypes between the two affected individuals. The affected 15-C4/MA shares the same haplotype as the unaffected 15-C3/FU, and the affected 15-C6/MA shares the same haplotype as the unaffecteds 15-C1/FU and 15-C5/FU. This data is consistent with exclusion from the chromosome 10p locus (Fig. 3.15), but requires confirmation using additional microsatellite markers.

### 3.5.2 CAVEOLIN 3 HAPLOTYPE ANALYSIS

In the non-dysferlin MM families haplotype analysis was also performed for the caveolin 3 region on chromosome 3. Four microsatellite markers mapping within and flanking the caveolin 3 gene were analysed: D3S3611, D3S3691, D3S1307 and D3S2170.

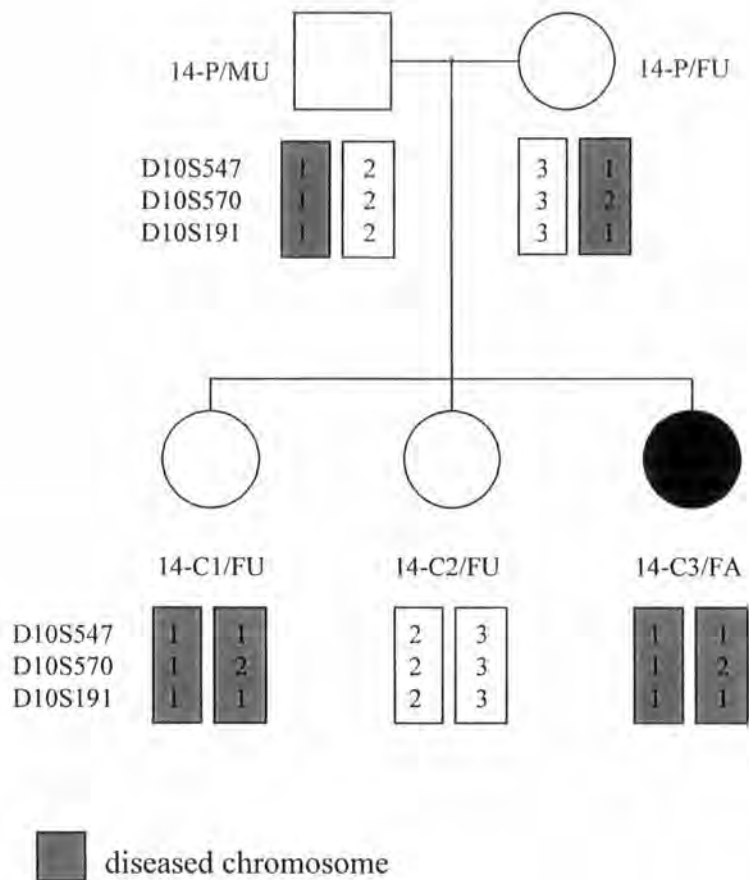
In Family 9, the markers D3S3611 and D3S3691 identify recombination in the affected individual. This haplotype is not shared by the unaffected sib, but the data is uninformative. Further analysis using additional microsatellite markers is necessary in order to establish linkage or exclusion (Fig. 3.16).

In Family 10, recombination of the paternal chromosome is seen in the affected at the marker D3S3611. Individual 10-C3/MU is also recombinant on the maternal chromosome at marker D3S1307. Although the affected does not share haplotype with either of the unaffecteds, the data is uninformative and analysis of further microsatellite markers is necessary to confirm linkage or exclusion (Fig. 3.17).



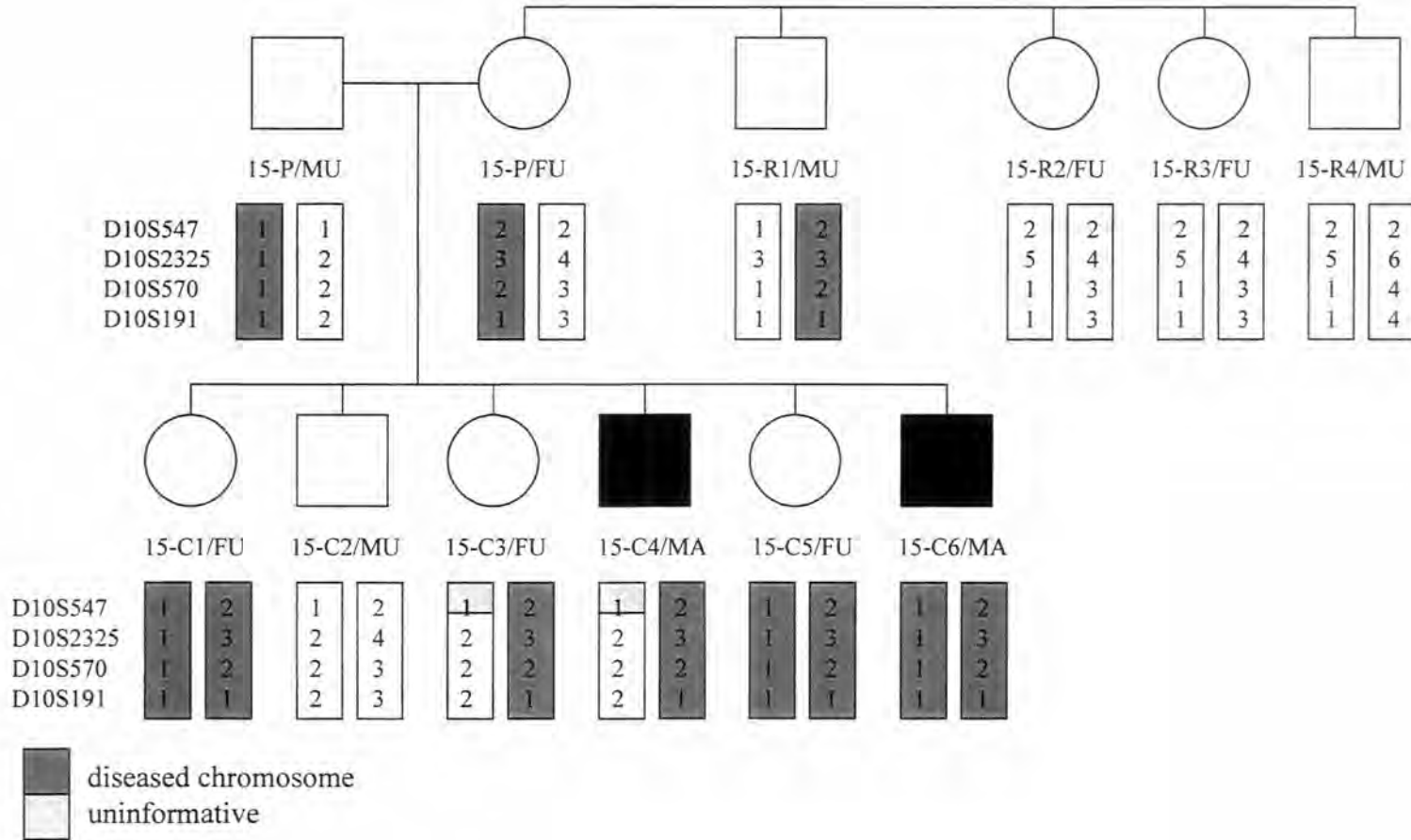
**Fig. 3.14 Chromosome 10p haplotyping in Family 14**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



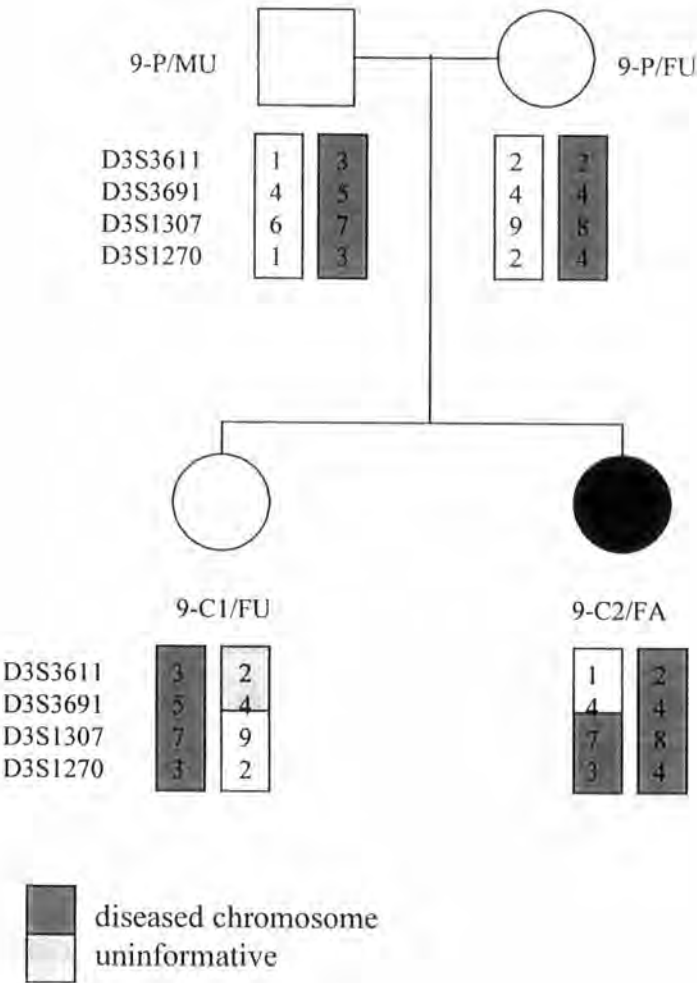
**Fig. 3.15 Chromosome 10p haplotyping in Family 15**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



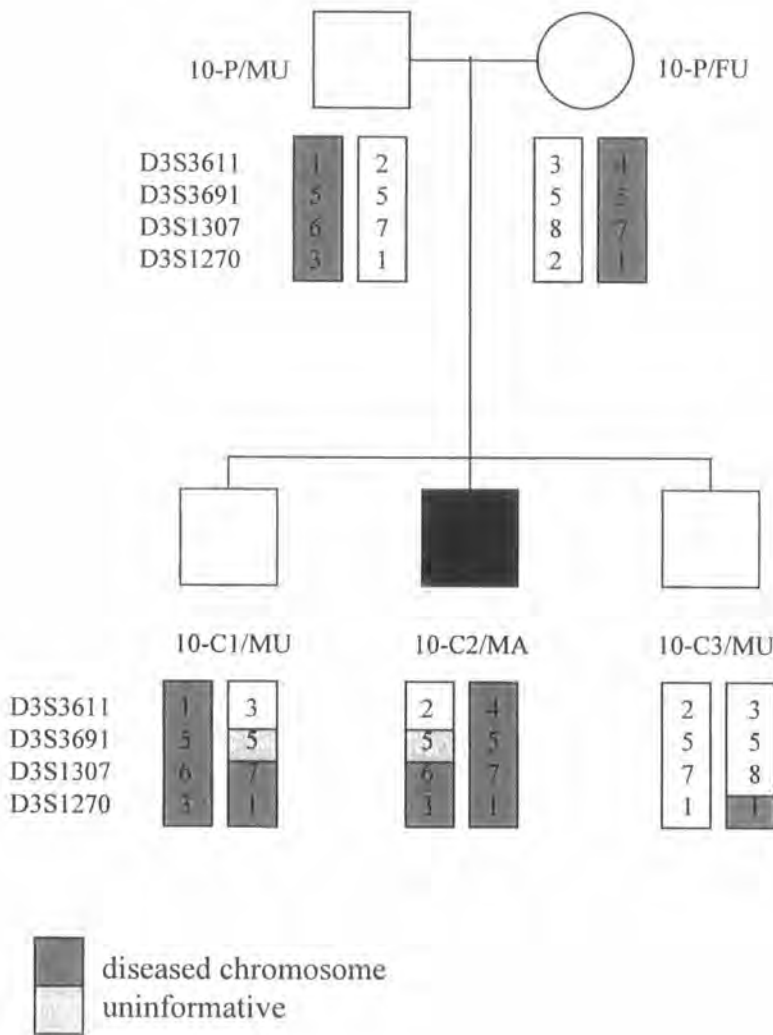
**Fig. 3.16** Chromosome 3 haplotyping in Family 9

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



**Fig. 3.17 Chromosome 3 haplotyping in Family 10**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



In Family 11, the two affecteds do not share the same haplotype. The affected individual 11-C3/FA shares haplotype with the unaffected 11-C5/MU. This data is consistent with exclusion (Fig. 3.18).

In Family 12, recombination is seen in the affected individual at marker D3S1270 on the maternal chromosome. This affected does not share haplotype with either of the unaffected sibs (Fig. 3.19). However the data is uninformative, and further analysis with additional microsatellite markers is necessary as in Family 10.

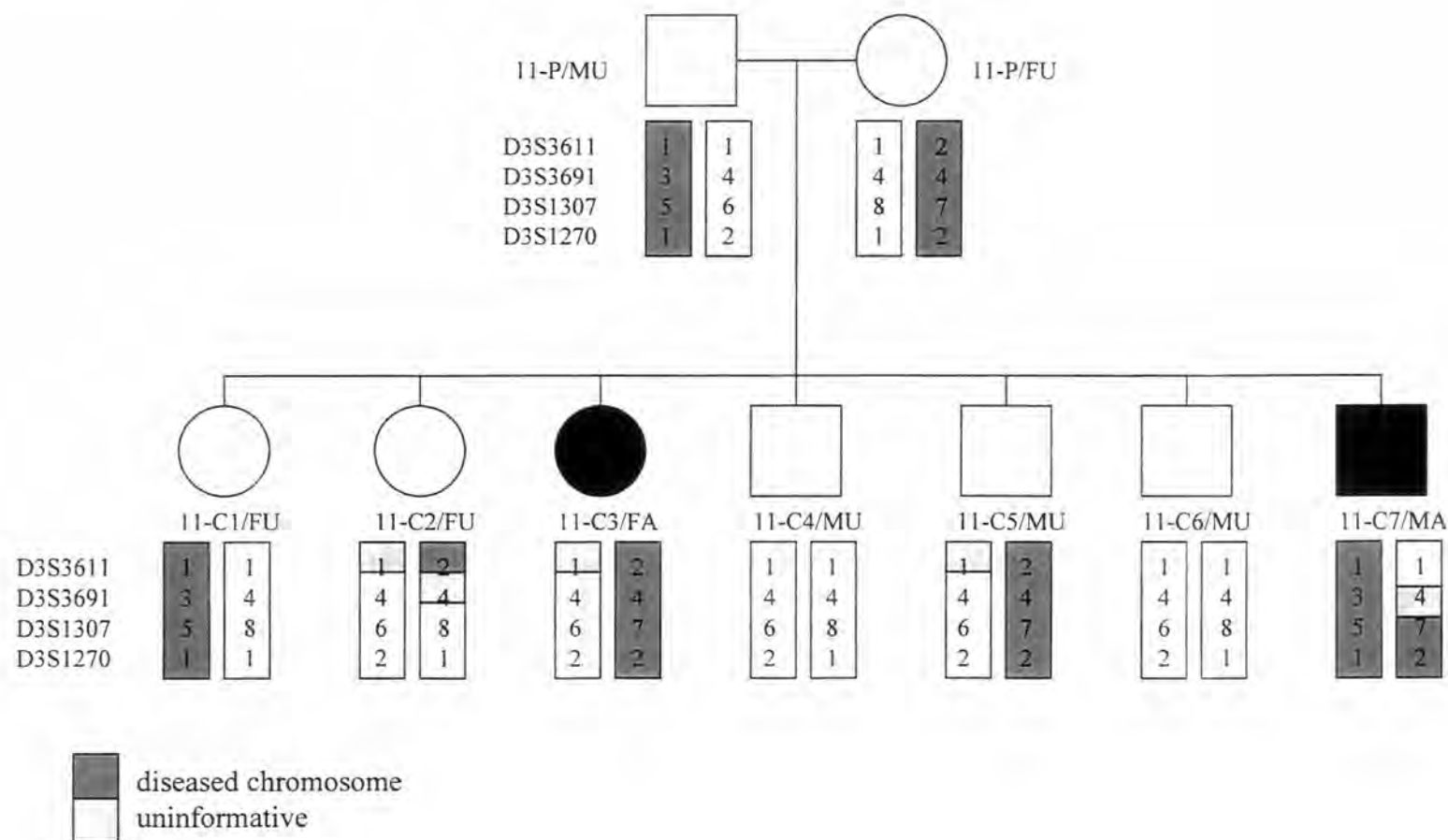
In Family 13, the two affecteds do not share haplotype. The affected individual 13-C2/MA shares haplotype with the unaffected 13-C4/FU. This data is consistent with exclusion from this locus (Fig.3.20).

In Family 15, the markers D3S1307 and D3S1270 identify recombinants by comparing the haplotypes of the two affecteds. The affected individual 15-C4/MA shares haplotype with the unaffecteds 15-C1/FU and 15-C3/FU, excluding this family from the locus on chromosome 3 (Fig. 3.21).

The gene was screened by SSCP and sequencing among the affecteds from each family. No mutations were identified.

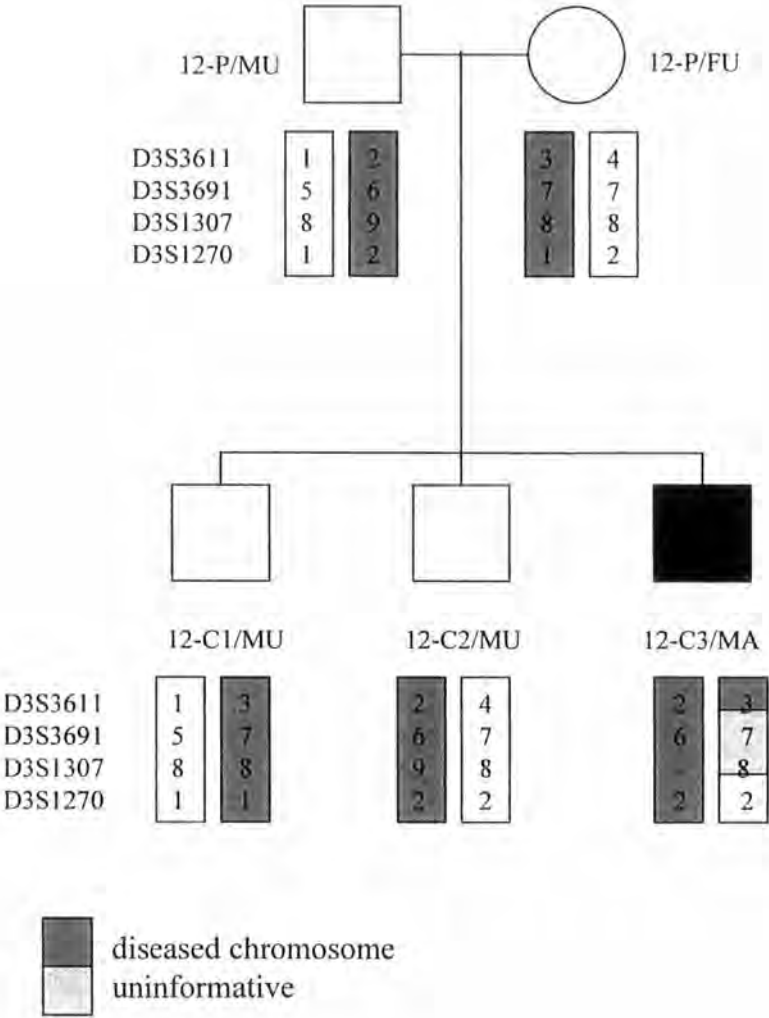
**Fig. 3.18 Chromosome 3 haplotyping in Family 11**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



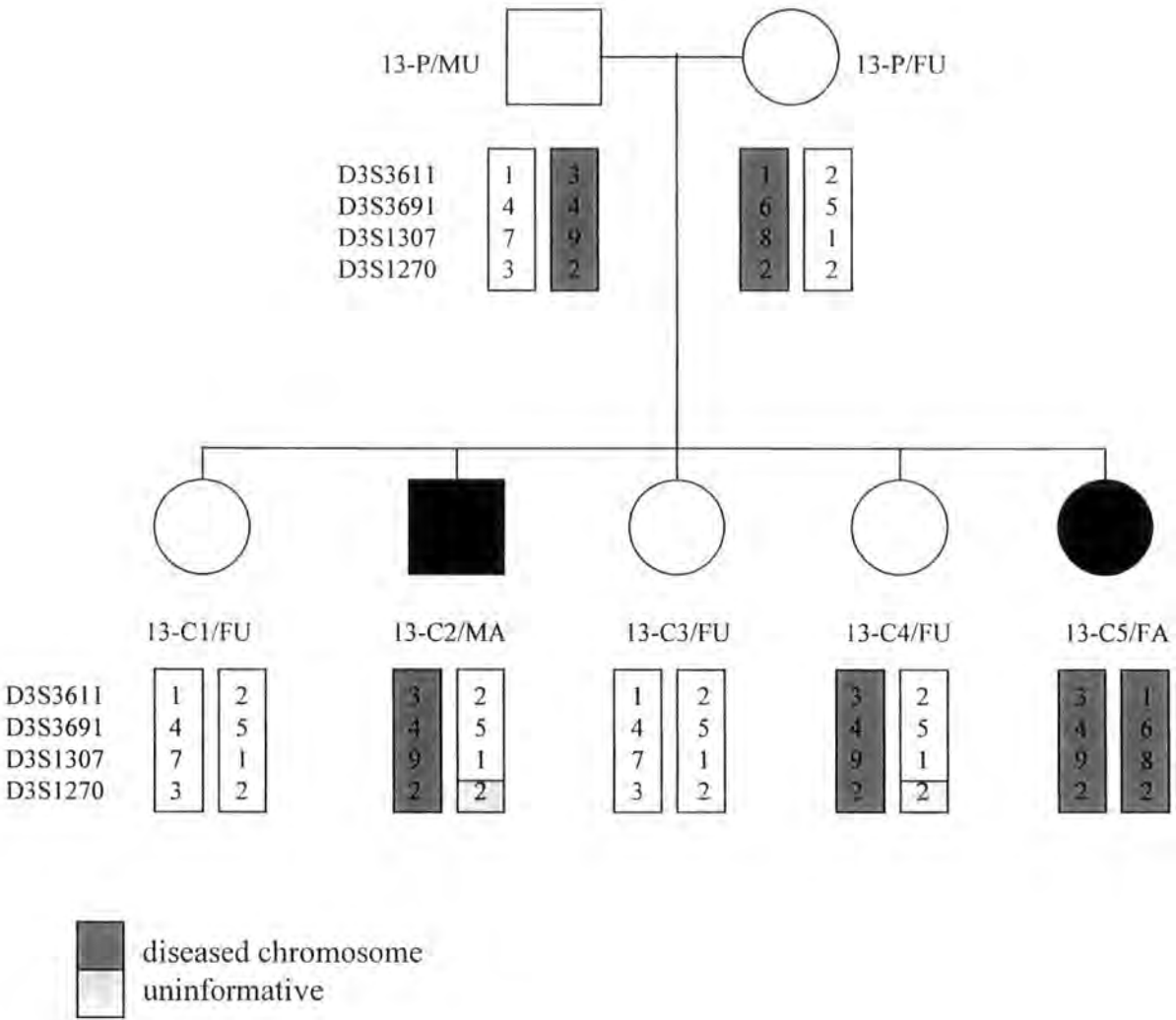
**Fig. 3.19** Chromosome 3 haplotyping in Family 12

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



**Fig 3.20 Chromosome 3 haplotyping in Family 13**

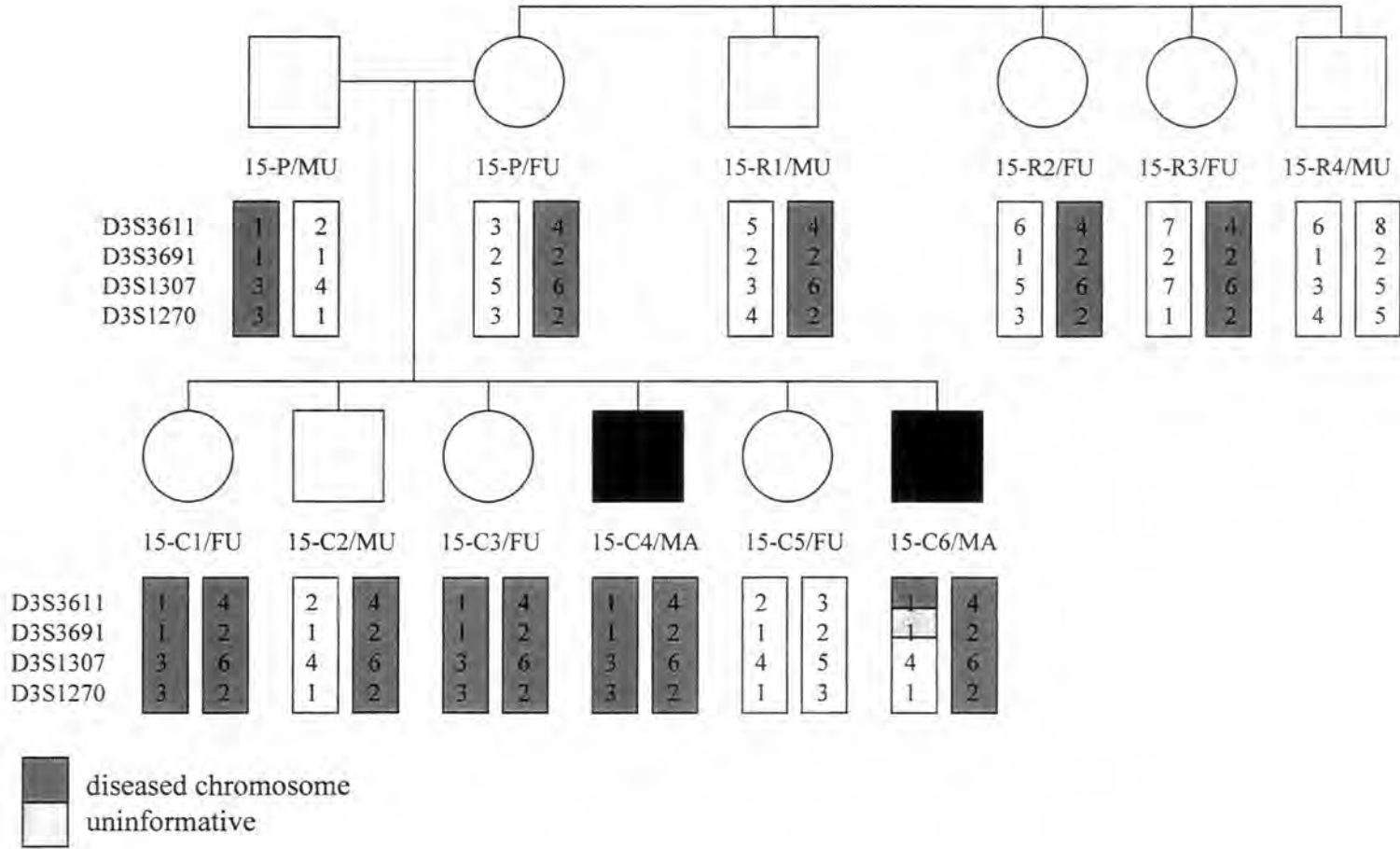
Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).





**Fig. 3.21 Chromosome 3 haplotyping in Family 15**

Affected individuals are represented by solid symbols and unaffected individuals by open symbols. Order of markers is given telomere → centromere (top → bottom).



## **CHAPTER 4**

### **DISCUSSION**

This study had two aims, firstly to work towards the identification of potential LGMD2I families and, by screening databases, to construct a physical map of the LGMD2I critical region and identify candidate genes mapping within this region. Secondly, we aimed to define the MMD2 region on chromosome 10 and to determine whether caveolin 3 is mutated in Myoshi Myopathy not linked to dysferlin, as well as to identify further genes involved in Myoshi Myopathy not linked to dysferlin.

We studied 6 potential LGMD2I families, 4 were suggestive of linkage. A primary physical map of the LGMD2I region was constructed, having at least 26 genes placed within the critical region between the centromeric marker D19S606 and the telomeric marker D19S879. Using LocusLink, candidate genes were identified on the basis of their potential role in muscular dystrophy. Four candidate genes, FXYD1, FLJ20200, FLJ10922 and SEPW1 were screened by SSCP analysis but no mutations were detected. However, during this time the LGMD2I gene was identified as the fukutin related protein gene (FKRP) (Brockington *et al.*, 2001a), by Francesco Muntoni's group in London.

The fukutin-related protein gene, FKRP, shares homology with fukutin, the protein product of the FCMD gene responsible for Fukuyama CMD (Kobayashi *et al.*, 1998). These proteins demonstrate sequence similarities to a family of proteins involved in modifying cell surface molecules (Aravind & Koonin, 1999), suggesting a role as a glycosyltransferase. All members of this family of proteins contain a conserved DxD motif found in many glycosyltransferases (Breton and Imberty, 1999). However it remains to be formally demonstrated that FKRP is itself a glycosyltransferase. The FKRP gene is also mutated in a severe form of CMD, MDC1C (Brockington *et al.*, 2001b). This disease is characterised by early onset, inability to achieve independent

ambulation, muscle hypertrophy, marked elevation of serum creatine kinase (CK), no brain involvement and a secondary deficiency of laminin  $\alpha 2$  (Brockington *et al.*, 2001b). Affected individuals also show a marked decrease in  $\alpha$ -dystroglycan expression as demonstrated by immunostaining and western blot analysis. The mutations in FKRP resulting in selective deficiency of  $\alpha$ -dystroglycan in skeletal muscle most likely occur due to abnormal glycosylation,  $\alpha$ -dystroglycan a heavily glycosylated peripheral membrane protein forming a link between the actin associated cytoskeleton and the extracellular matrix via laminin  $\alpha 2$  (Ibraghimov-Beskrovnaya *et al.*, 1992, Ervasti *et al.*, 1993).

Congenital muscular dystrophy (CMD) is also caused by mutations in the LAMA2 gene, encoding the laminin  $\alpha 2$  chain of merosin (Brockington *et al.*, 2001b). Around 40% of cases of congenital muscular dystrophy are due to LAMA2 mutations. Merosin is an extracellular matrix protein consisting of three laminin chains which form a link with  $\alpha 2$  between the peripheral membrane protein  $\alpha$ -dystroglycan and the basal lamina (Emery, AEH., ed. Neuromuscular disorders: Clinical and Molecular Genetics (Wiley, 1998) pp. 21-48). Children with mutations in LAMA2 usually have absent protein expression and a severe form of CMD, MDC1A. The clinical and genetic overlap of CMD and LGMD suggest that the underlying pathology of the diseases follow a similar pathway.

Following the cloning of the FKRP gene by Francesco Muntoni's group, the emphasis of this study then shifted from candidate gene analysis to the identification of FKRP mutations in linked families. Haplotype analysis revealed 3 of our 6 potential LGMD2I families to be suggestive of linkage; FKRP mutations were subsequently identified in all 3 families during Francesco Muntoni's study in London, as described in section 3.3.

During the investigation at London a total of 25 potential LGMD2I families were analysed; 14 of the families were consistent with linkage to the LGMD2I locus. Mutations were identified in individuals from 17 families by sequence analysis of the FKRP coding region. Unexpectedly, an identical C826A (Leu276Ileu) mutation was identified in affected individuals from 15 of the 17 families, and C826A has now been identified as the most common mutation found in any autosomal recessive muscular dystrophy in the North East of England (Brockington *et al.*, 2001a). Of the 15 individuals in whom the C826A mutation was identified, 5 were homozygous for the change, the remainder being compound heterozygotes. Of the 10 heterozygous individuals, the second allelic mutation was identified in four cases, these being two missense mutations (C934T, C947G), one nonsense mutation (390insTACC) and an in-frame deletion (426del12). Of the families without the C826A mutation, a heterozygous missense mutation (C427A) was identified in one case, and a homozygous missense mutation (G1016A) was identified in the case of one consanguineous family. In eight cases no mutations were found in the FKRP coding sequence, four of which were individuals from genetically informative families compatible with linkage to the FKRP locus. In 6 of the 10 compound heterozygotes for the C826A mutation, the second allelic mutation was not identified (Brockington *et al.*, 2001a). These findings strongly suggest that mutations also exist outside the FKRP coding region.

Collectively, the patients from the 25 LGMD families studied at London had a variable phenotype, though all were less severe than MDC1C where patients never acquired independent ambulation (Brockington *et al.*, 2001a). Five of the families had a severe LGMD phenotype, with onset of symptoms within the first two years of life. Despite the

overlap with MDC1C, the disease course was significantly milder than that of CMD and followed a Duchenne-like course in several aspects. The clinical features in these five families were characterised by delay in early motor milestones, waddling gait, difficulties in running, mild facial weakness, calf and occasionally tongue hypertrophy and Achilles tendon contractures. Serum CK was markedly elevated, with intelligence and brain MRI invariably normal. Three cases lost the ability to walk before the age of 14, four cases showed evidence of cardiac involvement (Brockington *et al.*, 2001a).

The remaining 12 families studied displayed a milder phenotype; all were non-consanguineous. Onset of symptoms was in the first decade of life for one patient, and in the second or third decade for the remaining patients, with early motor milestones being normal in all cases. Mild facial and neck flexion weakness was present in most patients, with calf hypertrophy and, less commonly, brachioradialis hypertrophy. Proximal muscles in both upper and lower limbs were weaker than distal muscles. There were no significant contractures, with the exception of mild reduction in lateral flexion and rotation of the neck in some patients. Progression of the disease followed a relatively benign course with all patients remaining ambulant, with the exception of one involved in an accident. Cardiac involvement was apparent in three of the families, and serum CK was also markedly elevated in these patients (between 10 and 30 times normal values). Intelligence and brain MRI were also normal among these patients (Brockington *et al.*, 2001a).

Patients at the severe end of the LGMD2I spectrum showed a mild deficiency of laminin  $\alpha 2$ , with patients having a milder phenotype showing normal expression of laminin  $\alpha 2$ . However in four patients a severe deficiency or total absence of the protein was

identified. This discrepancy suggests that mutations in FKRP result in the altered processing, epitope masking or abnormal folding of the laminin  $\alpha$ 2 polypeptide chain (Brockington *et al.*, 2001a).

The expression of  $\alpha$ -dystroglycan was abnormal in all muscle biopsies of LGMD2I patients available for study, an identical finding to MDC1C (Brockington *et al.*, 2001a).

Genotype-phenotype correlations are now emerging, with most patients having a homozygous C826A mutation following a relatively mild LGMD disease course (Brockington *et al.*, 2001a). Patients heterozygous for the change on the whole follow a more severe clinical course, though less severe than MDC1C. Missense mutations in the FKRP gene are associated with the MDC1C phenotype, but no null mutations are observed in these patients, suggesting that these mutations are embryonic lethal. MDC1C, along with the congenital muscular dystrophies MDC1A and MDC1B, share clinical features with three other myopathies, Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (WWS) (Emery, AEH., ed. Neuromuscular disorders: Clinical and Molecular Genetics (Wiley, 1998) pp. 21-48). The genes for these diseases have also now been predicted to be glycosyltransferases, as all show deficiency of  $\alpha$ -dystroglycan (Hayashi *et al.*, 2001, Kano *et al.*, 2002, Michele *et al.*, 2002). Recently, the gene involved in Walker Warburg syndrome, POMT1, has been shown to be an O-mannosyltransferase (Beltran-Valero De Bernabe *et al.*, 2002), and the gene POMGnT1 involved in MEB has been shown to be an O-mannoside N-acetylglucosaminyltransferase (Yoshida *et al.*, 2001). The congenital muscular dystrophies are divided into two groups, merosin-deficient CMD and merosin-positive CMD. Mutation in the merosin gene causes complete loss of the protein,

resulting in a severe muscular dystrophy phenotype (Voit, 1998). In contrast, with non-merosin linked CMD the presence of the protein merosin results in a milder clinical course (Voit, 1998).

The defining of mutations and the establishment of such genotype-phenotype correlations translates into a greater understanding of prognostic and treatment implications, as well as opening potential for accurate genetic counselling. Thus abnormal protein glycosylation is emerging to be a common pathomechanism in muscular dystrophy. Protein glycosylation occurs in the endoplasmic reticulum and the Golgi apparatus. Proteins having roles in cell-cell adhesion are shown to be heavily glycosylated (Kobata *et al.*, 1992). Proteins can be grouped into N-glycans and O-glycans depending upon the amino acid residue which is glycosylated (Martin-Rendon and Blake, 2003). O-glycosylation involves serine and threonine residues, N-glycosylation involving asparagine residues. The different sugars added impact upon the structure and function of the protein (Breton and Imberty, 1999). O-mannose linked glycosylation is high in the brain, peripheral nerve and muscle tissue, with 30% of O-linked sugar chains in the brain being O-mannose (Van den Steen, *et al.*, 1998).  $\alpha$ -Dystroglycan is an O-mannose linked protein, which goes some way towards explaining the occurrence of complex brain and eye abnormalities in CMD patients.

Mutations in dysferlin are responsible for the muscular dystrophies LGMD2B and Miyoshi Myopathy (MM) (Bashir *et al.*, 1998, Liu *et al.*, 1998). The dysferlin gene encodes a novel protein which shares homology with the *Caenorhabditis-elegans* sperm vesicle fusion protein FER-1 (Achanzar, W. E. and Ward, S., 1997). Although the function of the dysferlin protein is unknown, it has been shown to be a membrane protein



(Moss *et al.*, 1999; Matsuda *et al.*, 1999) which is characterised by the presence of tandem C2 domains and is predicted to have a role in membrane trafficking (Liu *et al.*, 1998; Bashir *et al.*, 1998). The identification of this gene has provided a molecular test, allowing the identification of cases of Miyoshi Myopathy which are not linked to dysferlin. In this study we have worked towards identifying genes involved in MM not linked to dysferlin, which will ultimately serve to increase our understanding of the function of dysferlin.

Pathological studies have recently shown that dysferlin deficient muscle has lesions of the cell membrane, with evidence of repair (Selcen *et al.*, 2001) and abnormal accumulation of subsarcolemmal vesicles (Piccolo *et al.*, 2000, Fanin *et al.*, 2001). In dysferlin deficient MM, muscle membrane repair has been shown to be defective (Bansal *et al.*, 2003). In normal muscle, dysferlin is enriched at the site of a wound (Bansal *et al.*, 2003), suggesting that it plays a role in cell membrane repair. Repair of muscle cell membrane involves the fusion of lysosomes with the plasma membrane upon elevation of intracellular calcium (Reddy *et al.*, 2001, McNeil, 2002). Vesicles fuse with the membrane and with each other to form a patch over the wound site, and dysferlin is thought to be involved in the formation of this patch membrane and its addition to the wound site (McNeil, 2002).

Our approach to identify genes responsible for non-dysferlin MM was concentrated on evidence of linkage to chromosome 10 (Linssen *et al.*, 1998), and on the region on chromosome 3 to which caveolin 3 maps. Since caveolin 3 has been shown to co-immunoprecipitate with dysferlin (Matsuda *et al.*, 2001), and mutations in caveolin 3 cause several muscular dystrophies including hyperCKemia (Merlini *et al.*, 2002),

rippling muscle disease Kubisch *et al.*, 2003), distal myopathy (Tateyama *et al.*, 2002) and LGMD1C (Minetti *et al.*, 1998), we considered caveolin 3 to be a candidate for non-dysferlin MM.

Two of our families showed tentative linkage to chromosome 10, and although the LOD score obtained for these families was not significant, it was close to +3. Further genetic analysis performed on these families identified a region of 25Mb which is shared by four affecteds in two families, with break points being defined. Such a large non-recombinant region provides further evidence for the existence of an MMD2 gene on chromosome 10. Our studies have identified the boundaries of this gene, and should now allow the identification of candidate genes for mutation screening. In smaller families haplotype analysis was performed in order to identify further families consistent with linkage to the chromosome 10 locus, but the results were not informative. Further work would need to be carried out using additional microsatellite markers in order to confirm linkage or exclusion in these families.

Four microsatellite markers mapping within and flanking the region on chromosome 3 to which caveolin 3 maps were analysed in MM families not suggestive of linkage to chromosome 10. For some families the data was suggestive of linkage, but on the whole the data was uninformative. Mutation screening of the CAV3 gene was performed for these families by a student, and followed up by DNA sequencing, but no mutations were identified. This data suggests that caveolin 3 is not implicated in these cases of Miyoshi Myopathy.

Data is emerging on the role of dysferlin in membrane repair, and recently other proteins interacting with dysferlin have been identified, including annexin A1 and annexin A2

(Lennon *et al.*, 2003), which are calcium binding proteins. This new information provides further candidates for haplotype analysis and mutation screening. In our MM families not linked to chromosome 10, work is continuing to assess the candidacy of these proteins.

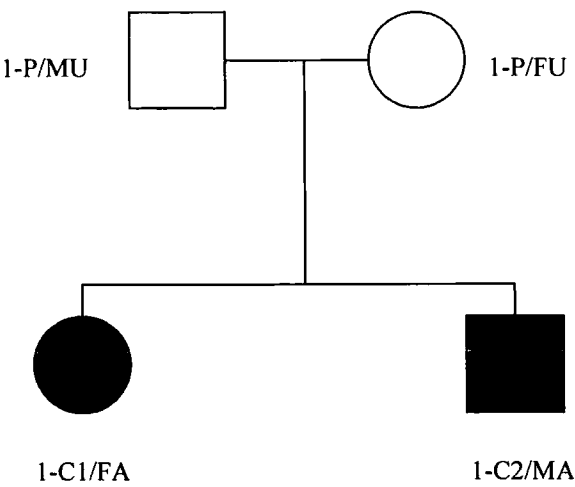
X genes have now been shown to be involved in muscular dystrophy, with overlaps such as that seen in LGMD and CMD emerging (Vainzof and Zatz, 2003). Mechanisms involved in muscle membrane stability and repair, sarcomere assembly and muscle cell signaling are being identified, which have helped to highlight the different mechanisms by which muscular dystrophy can arise (Vainzof and Zatz, 2003).

My work in this project has helped to emphasize the role of glycosylation in muscular dystrophy through our work on the LGMD2I region on chromosome 19, and continuing work on our Miyoshi Myopathy families not linked to dysferlin will lead to the identification of other genes which are functioning in the dysferlin membrane repair pathway.

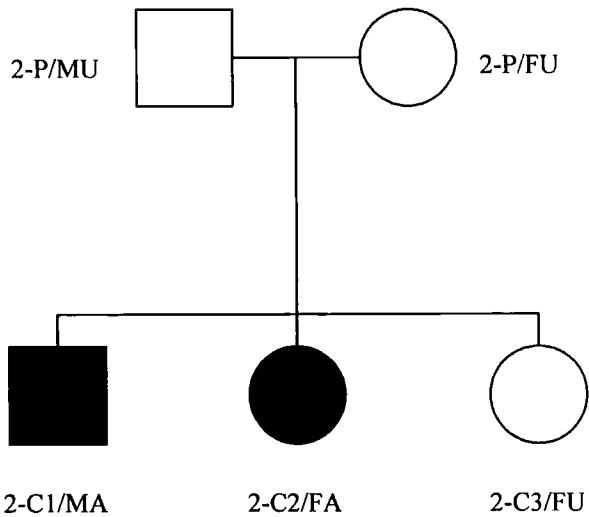
## **APPENDIX**

**Pedigrees of 15 families available for haplotyping**

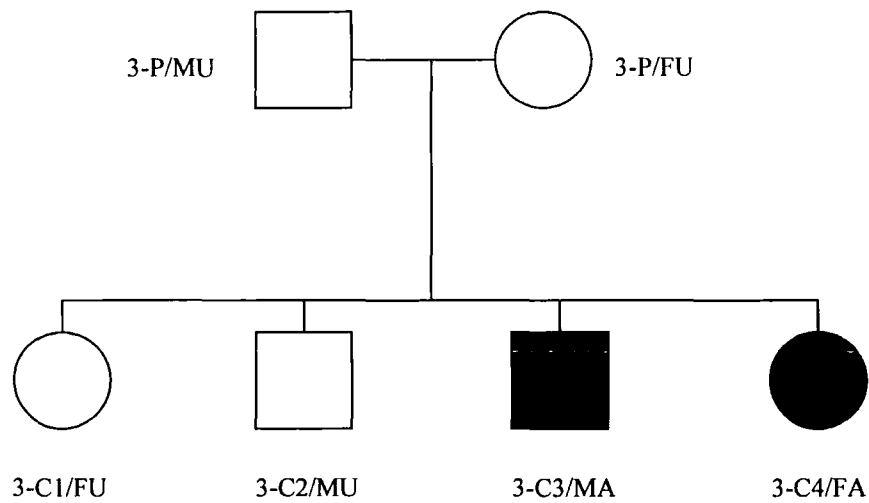
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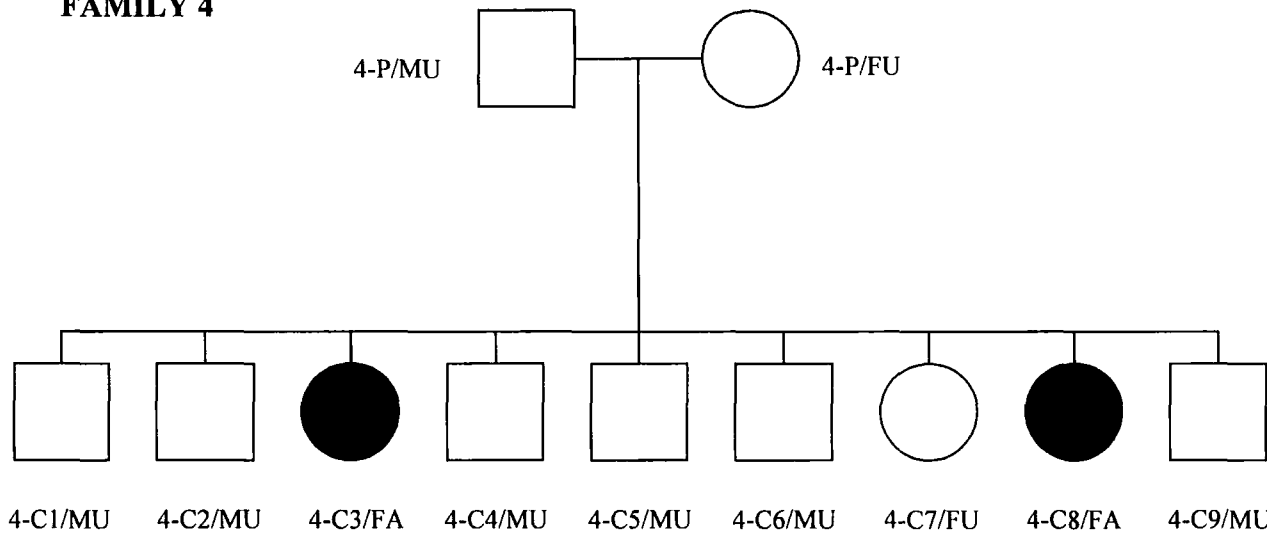
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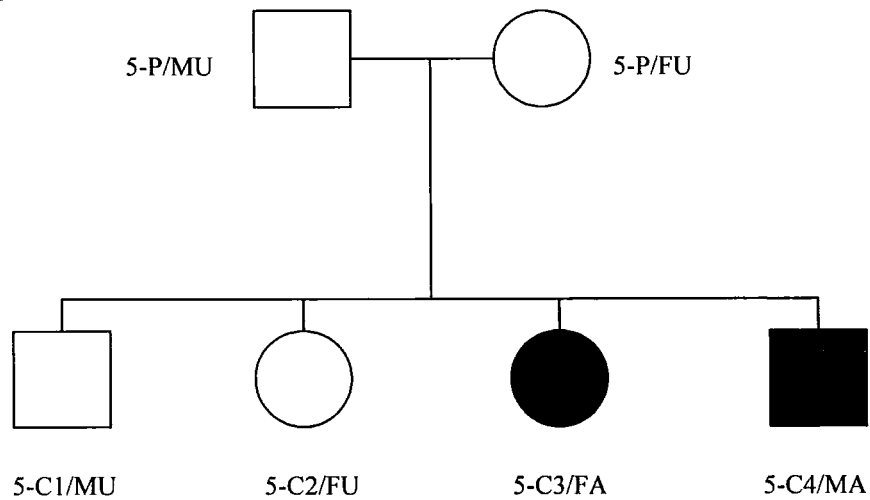
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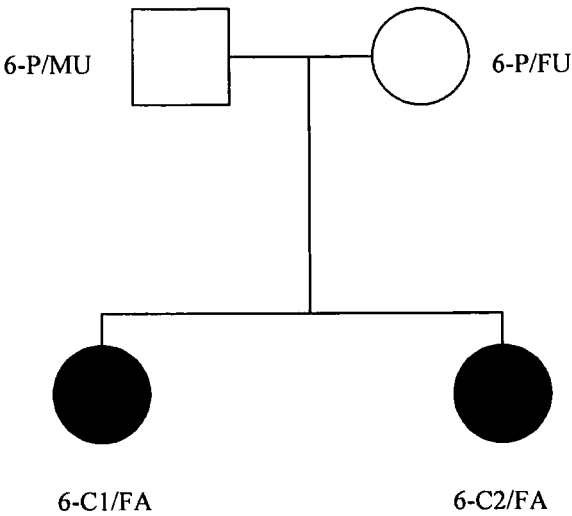
**FAMILY 4**



**FAMILY 5**

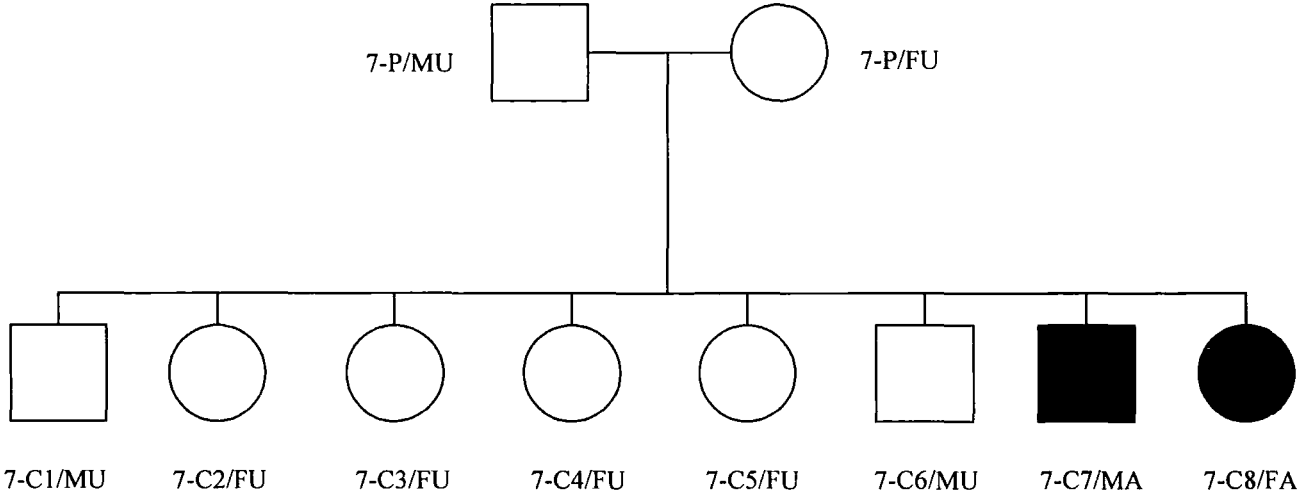


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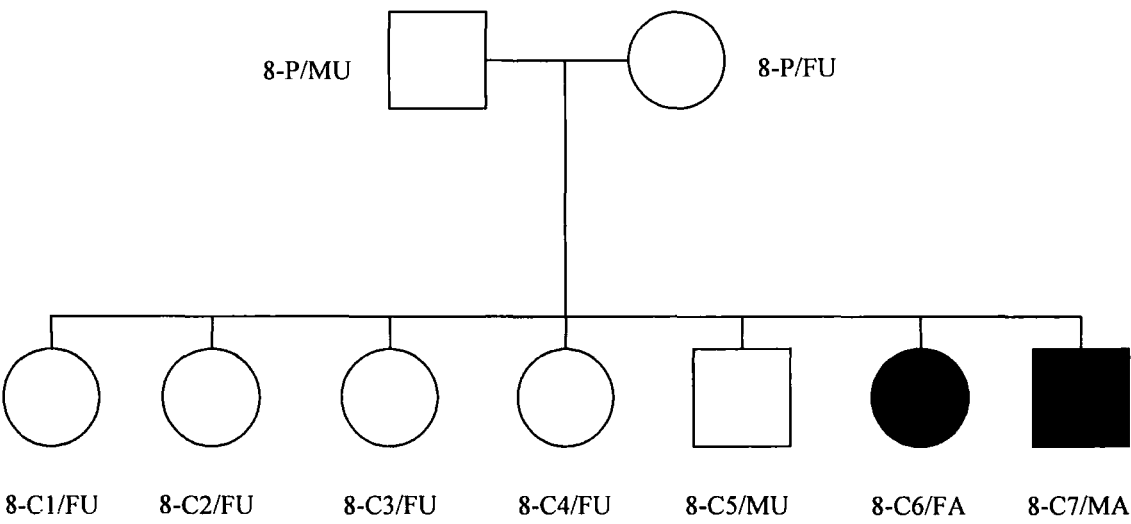




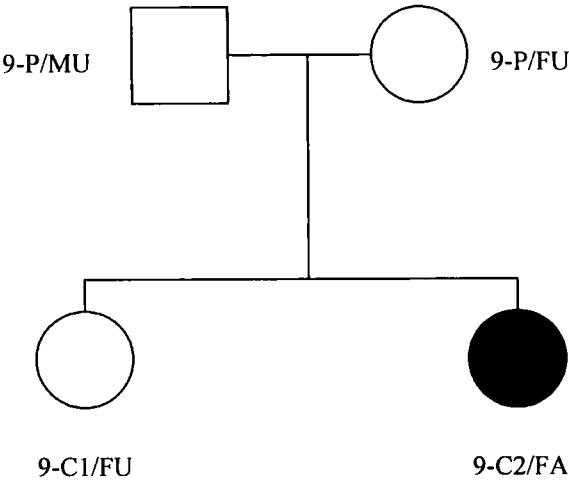
**FAMILY 7**



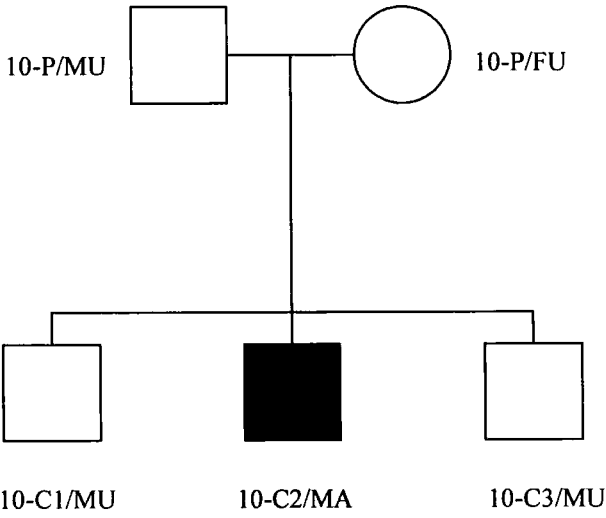
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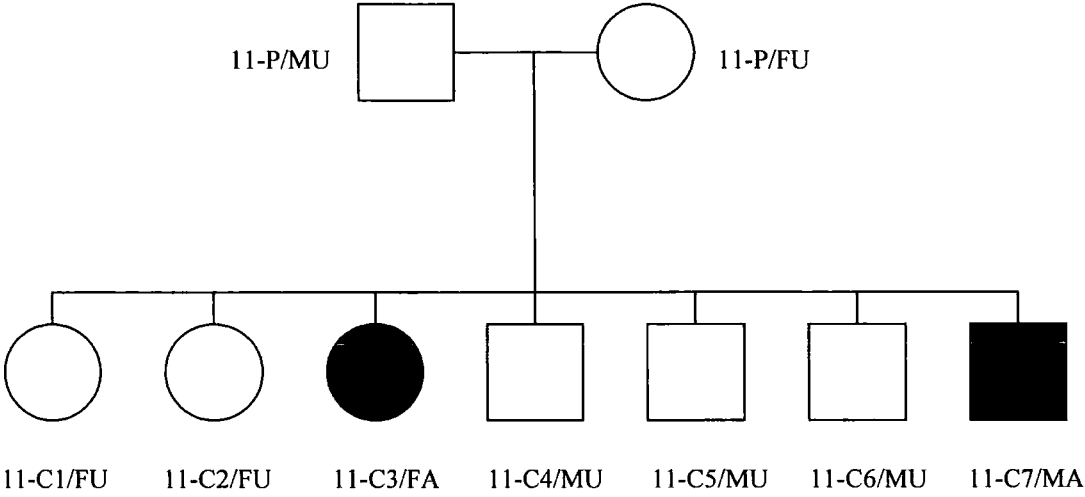
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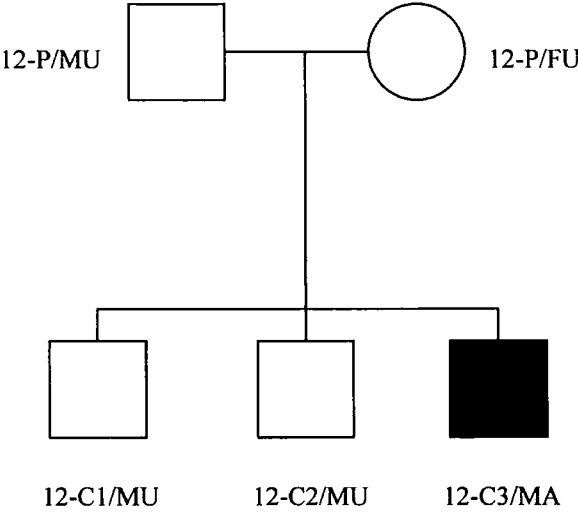
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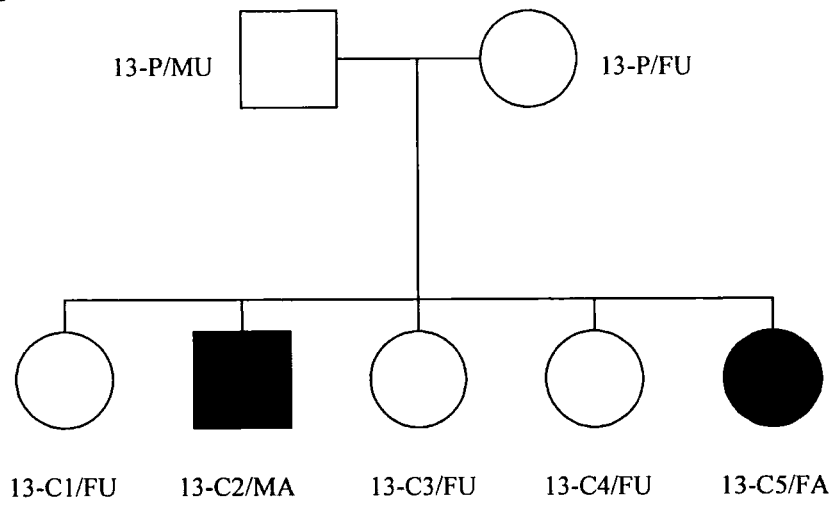
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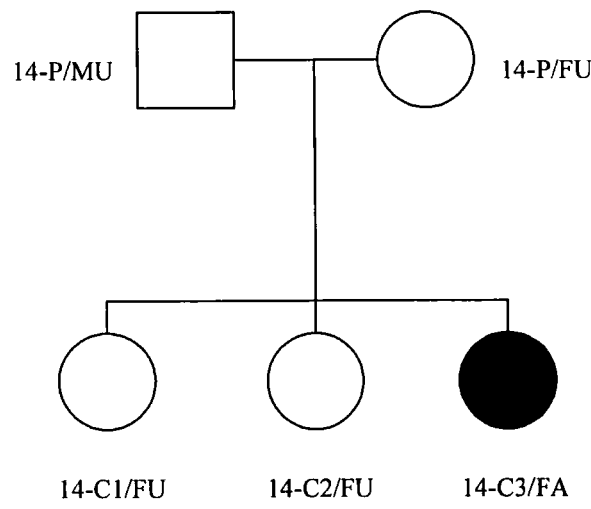
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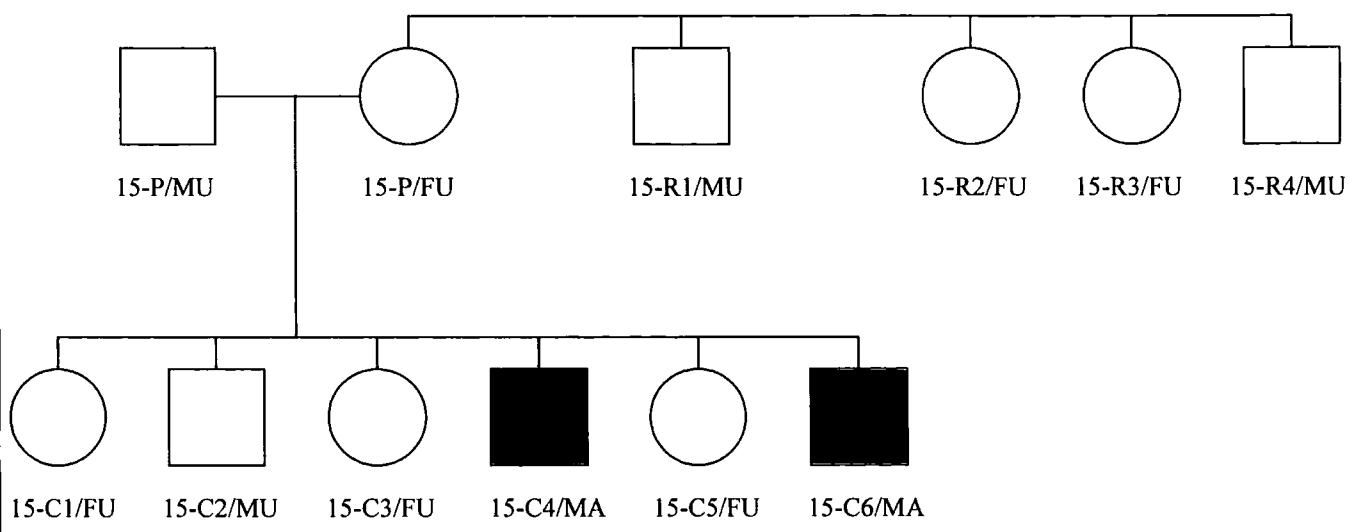
**FAMILY 13**



**FAMILY 14**



FAMILY 15



**Mutations in the fukutin-related protein gene (FKRP) identify  
limb girdle muscular dystrophy 2I as a milder allelic variant  
of congenital muscular dystrophy MDC1C.**

**Brockington *et al.*, 2001a**

***Hum Mol Genet* 2001; 10(25): 2851-2859**

## ARTICLE

# Mutations in the fukutin-related protein gene (*FKRP*) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C

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The limb girdle and congenital muscular dystrophies (LGMD and CMD) are characterized by skeletal muscle weakness and dystrophic muscle changes. The onset of symptoms in CMD is within the first few months of life, whereas in LGMD they can occur in late childhood, adolescence or adult life. We have recently demonstrated that the fukutin-related protein gene (*FKRP*) is mutated in a severe form of CMD (MDC1C), characterized by the inability to walk, leg muscle hypertrophy and a secondary deficiency of laminin  $\alpha 2$  and  $\alpha$ -dystroglycan. Both MDC1C and LGMD2I map to an identical region on chromosome 19q13.3. To investigate whether these are allelic disorders, we undertook mutation analysis of *FKRP* in 25 potential LGMD2I families, including some with a severe and early onset phenotype. Mutations were identified in individuals from 17 families. A variable reduction of  $\alpha$ -dystroglycan expression was observed in the skeletal muscle biopsy of all individuals studied. In addition, several cases showed a deficiency of laminin  $\alpha 2$  either by immunocytochemistry or western blotting. Unexpectedly, affected individuals from 15 families had an identical C826A (Leu276Ileu) mutation, including five that were homozygous for this change. Linkage analysis identified at least two possible haplotypes in linkage disequilibrium with this mutation. Patients with the C826A change had the clinically less severe LGMD2I phenotype, suggesting that this is a less disruptive *FKRP* mutation than those found in MDC1C. The spectrum of LGMD2I phenotypes ranged from infants with an early presentation and a Duchenne-like disease course including cardiomyopathy, to milder phenotypes compatible with a favourable long-term outcome.

## INTRODUCTION

The progressive muscle degeneration that gives rise to dystrophic changes on skeletal muscle biopsy is found in a variety of clinically and genetically defined conditions. Two

common forms of muscular dystrophy, distinguishable by their age of onset, are the congenital muscular dystrophies (CMDs) and the limb-girdle muscular dystrophies (LGMDs). In CMD, onset of symptoms is at birth or within the first 6 months of life (1), whereas in LGMD, they can occur in late childhood,

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Table 1. Summary of the clinical features of LGMD2I patients

Family	Sex	Age (years)	Age onset (years)	First symptom	Serum CK	Muscle hypertrophy	Weakest muscle	Cardiomyopathy	Wheelchair bound	Laminin $\alpha$ 2	$\alpha$ -Dystroglycan
1	M	6	<0.5	Hypotonia, DMM	>1000	Calf, legs	Hip girdle	No	No	Normal (I)	ND
2	F	10	1	Hypotonia, DMM	3160	Tongue	Neck, shoulder	Borderline LVF	No	Reduced (I)	Severely reduced
	M	18	1.5	Waddle	2850	Leg, calves, tongue	Hip = shoulder girdle	Severe LV, hypokinesia	14	ND	ND
	M	11	1.5	Waddle	2328	Leg, calves, tongue	Hip = shoulder girdle	ND	No	ND	ND
	M	19	1.5	Waddle	1700–3300	Leg, tongue	Hip = shoulder girdle	Moderate LV, hypokinesia	12	ND	Severely reduced
	F	20	2	Waddle	3160	Leg, calves	Deltoid	LVD	13	Reduced (I)	Severely reduced
	F	10	2.5	Toe walking, fatigue	1380–7795	Leg, calves	Deltoid	ND	No	Reduced (I)	Reduced
	F	10	2.5	DMM	1278	No	Hip girdle	No	No	Reduced (I)	ND
	M	28	4	Toe walking, no running	4105	No	Hip girdle	Moderate LV, hypokinesia	No	ND	ND
	M	22	4	No running	ND	No	Hip girdle	ND	No	ND	ND
	F	37	7	Waddle, arm weakness	1150	Tongue	Shoulder girdle	No	12	ND	ND
	M	NA	NA	NA	NA	NA	NA	NA	NA	ND	ND
	F	17	9	Gowers	2910–8200	Calf, legs	Neck, deltoid	Borderline LVF	No	Reduced (I)	Severely reduced
	F	18	9	Stairs	>1000	No	Tibialis anterior	No	No	ND	ND
	M	NA	NA	NA	NA	NA	NA	NA	NA	ND	ND
	M	16	13	Gowers	1305–6820	Leg, calves	Deltoid	ND	No	Reduced (I)	Severely reduced
	F	37	27	Stairs	2870	Calf	Hip girdle	No	Part-time, 35	Reduced (WB)	Reduced
	F	39	28	Stairs	2300	Calf, brachioradialis	Triceps, hip girdle	ND	No	Reduced (WB)	ND
	F	46	40	Stairs, running	2230	Calf, brachioradialis	Hip girdle	No	No	Reduced (WB)	Reduced
	M	48	35	Stairs	1123	Brachioradialis	Hip girdle	No	No	Reduced, (WB)	ND
	M	37	29	Stairs	>1000	Calf, brachioradialis	Hip girdle	No	Part-time, 36	Reduced (WB)	Reduced
	F	27	23	Stairs	4210	Leg, calves	Hip = shoulder girdle	No	No	Reduced (I)	Reduced

western blot; ND, no data; NA, not available; LVF, left ventricular function; LV, left ventricular.

adulthood or even adult life (2,3). Both CMD and LGMD are highly heterogeneous diseases. Inheritance in LGMD can be either autosomal dominant (LGMD type 1) or autosomal recessive (LGMD type 2), whereas CMD is always recessively inherited. To date, at least 14 types of LGMD have been reported and though the majority of causative genes have been found to encode proteins associated with the sarcolemma, genes encoding cytoskeletal proteins and muscle-specific myofibrillar proteins have also been implicated (2). Fewer disease genes have been identified in CMD (reviewed in 4). Mutations in the *LAMA2* gene on chromosome 6q22–23, encoding the laminin chain of merosin (laminin-2), were identified in 1994 and account for ~40% of CMD cases (5–7). Merosin is an extra-

cellular matrix protein that consists of three laminin chains,  $\alpha$ 2 $\beta$ 1 $\gamma$ 1, with  $\alpha$ 2 forming a link between the peripheral membrane protein  $\alpha$ -dystroglycan and the basal lamina. Children with mutations in the *LAMA2* gene usually have absent protein expression and a severe form of CMD, commonly referred to as merosin-deficient CMD or MDC1A.

It is now clear, both clinically and genetically, that CMD and LGMD can overlap, suggesting that the underlying pathology in these diseases may follow a similar pathway. We have previously described a large Turkish kindred, with a clear LGMD phenotype, linked to the *LAMA2* locus (8). Patients had a reduction in the expression of laminin  $\alpha$ 2 in their skeletal muscle biopsy. More recently, mutations in the *LAMA2* gene in



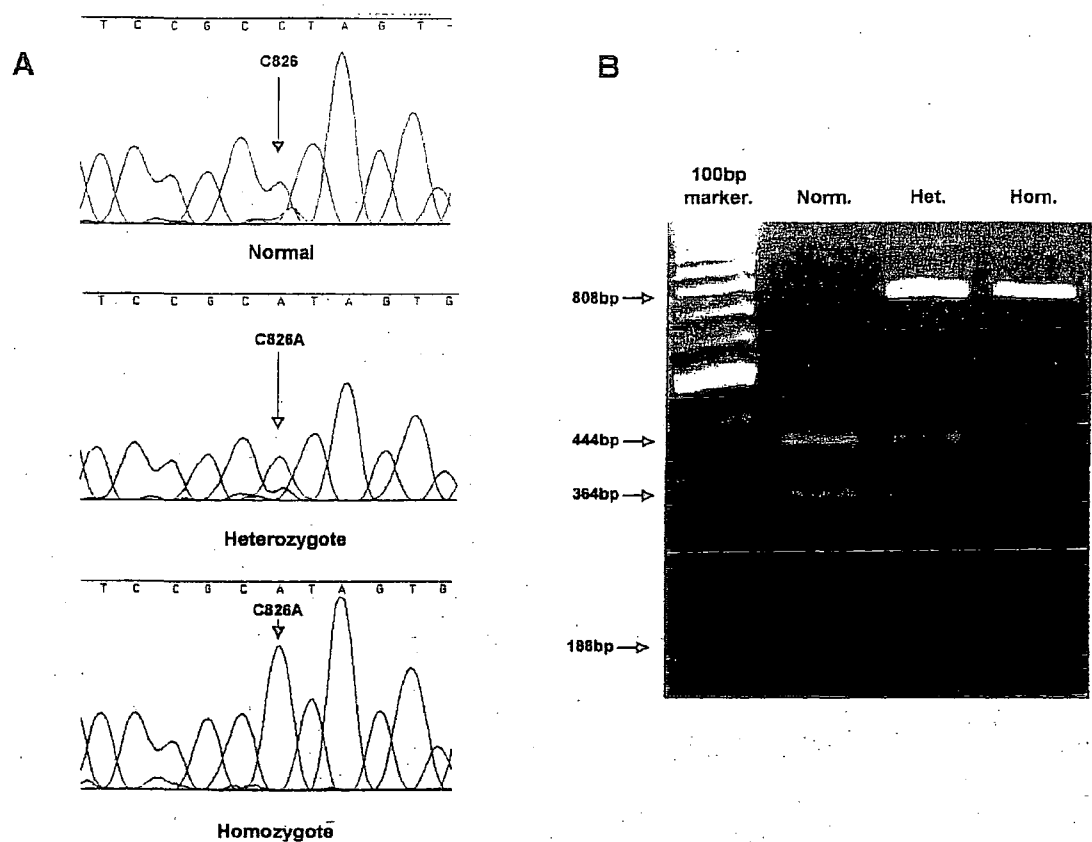


Figure 1. (A) Sequencing chromatograms showing the C826A mutation. (B) Restriction enzyme analysis of the C826A mutation. A 996 bp PCR fragment was digested with *Bfa*I. Wild-type DNA is cut into three fragments of 445, 364 and 189 bp, whereas products containing the mutation, which introduces the loss of a *Bfa*I site, are cleaved into two fragments of 808 and 189 bp. Norm., wild-type DNA; Het., heterozygote; Hom., homozygote.

Table 2. Summary of *FKRP* mutations in LGMDI

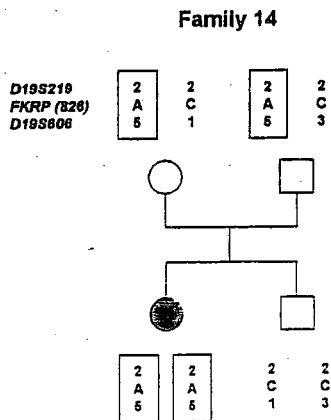
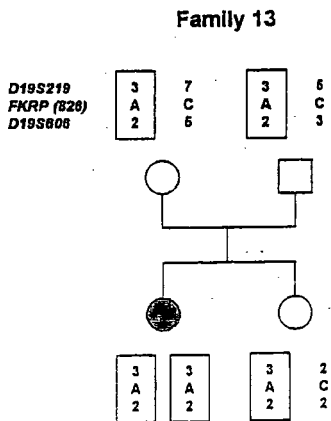
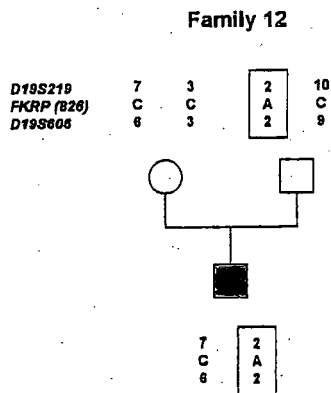
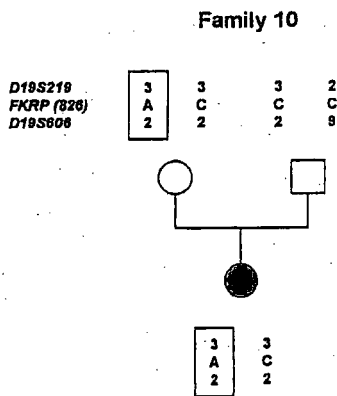
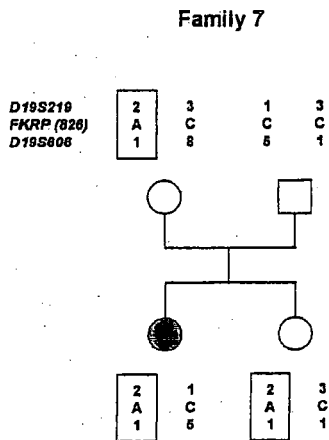
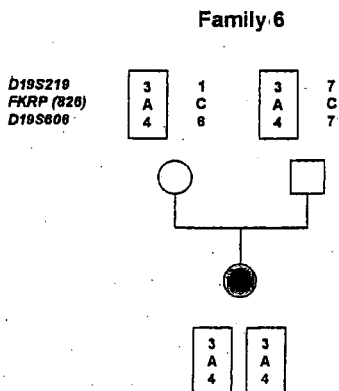
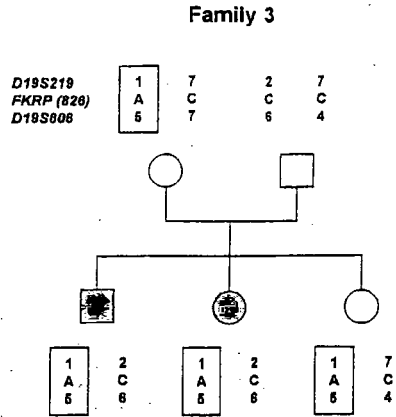
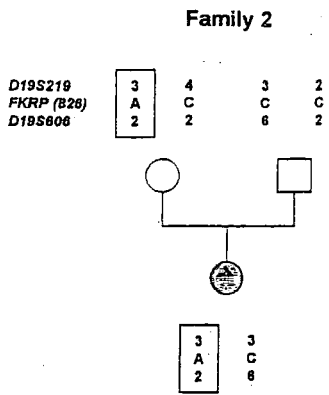
Family	Change	Protein effect	Mutational status
6, 8, 13, 14, 15	C826A	Leu276Ile	Homozygous
1	G1016T	Arg339Leu	Homozygous
3, 4, 5, 12, 16, 17	C826A	Leu276Ile	Compound
	?		Heterozygote
2	C826A	Leu276Ile	Compound
	390insTACC	Gly132Stop	Heterozygote
9	C826A	Leu276Ile	Compound
	C934T	Arg312Cys	Heterozygote
7	C826A	Leu276Ile	Compound
	C947G	Pro316Arg	Heterozygote
10	C826A	Leu276Ile	Compound
	426del12nt	143delRMVE	Heterozygote
11	C427A	Arg143Ser	Compound
	?		Heterozygote

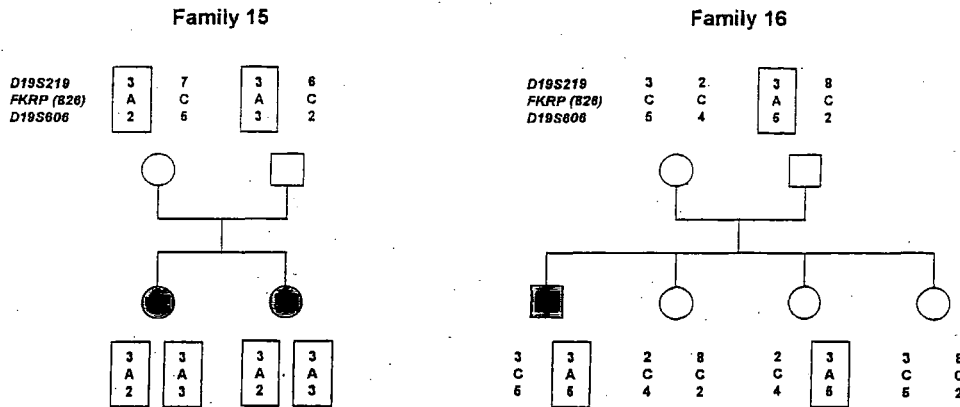
?, second allelic mutation unidentified.

several patients with a LGMD phenotype and a partial laminin  $\alpha$ 2 deficiency have been identified (9–11), confirming that

mutations in this gene can result in either a severe disease (MDC1A) or a mild LGMD-like disorder, depending on the type and location of the mutation within the gene.

A number of CMD forms have been described that have a reduction in laminin  $\alpha$ 2 expression not due to mutations in the *LAMA2* gene (secondary laminin  $\alpha$ 2 deficiency). One of these is Fukuyama CMD (FCMD; MIM 253800), a multi-system disease in which brain, skeletal and cardiac muscle are affected (12). The *FCMD* gene encodes fukutin, a protein of unknown function (13). A profound depletion of skeletal and cardiac muscle  $\alpha$ -dystroglycan has recently been reported in FCMD (14). We have recently identified a new member of the fukutin protein family, *FKRP* (15) (fukutin-related protein). Analysis of both *FKRP* and fukutin demonstrates sequence similarities to a family of proteins involved in modifying cell surface molecules, such as glycoproteins and glycolipids (16). All members of this family of proteins contain a conserved DxD motif found in many glycosyltransferases (17), though formal proof that *FKRP* is itself a glycosyltransferase remains to be demonstrated. Both proteins also have a putative type II membrane spanning a region consistent with localization to the Golgi apparatus, though it has been suggested that fukutin is also secreted (13). The *FKRP* gene is mutated in a severe form of CMD (MDC1C), characterized by early onset, inability to achieve independent ambulation, muscle hypertrophy, marked elevation of serum creatine kinases (CK), no brain involvement





**Figure 2** (above and opposite). The pedigrees of 10 LGMD2I families showing the inheritance of the C826A mutation. The haplotypes containing the mutation are boxed. Markers *D19S219* and *D19S606* are based 3 cM apart on the Genethon map. *D19S606* is <500 kb away from *FKRP* according to the latest sequence maps.

and a secondary deficiency of laminin  $\alpha 2$  (15). In addition, affected individuals had a marked decrease in immunostaining of muscle  $\alpha$ -dystroglycan and a reduction in its molecular weight on western blot analysis. We have suggested the abnormal expression of  $\alpha$ -dystroglycan is most likely a result of its altered glycosylation.

The *FKRP* gene maps to an identical region on chromosome 19q13.3 as LGMD2I (17), between markers *D19S219* and *D19S606*, suggesting that MDC1C and LGMD2I may be allelic disorders. To test this hypothesis we collected 25 LGMD families, including 14 that were consistent with links to the LGMD2I locus. Mutation analysis of the *FKRP* gene identified mutations in 17 families. In patients where muscle was available, a secondary laminin  $\alpha 2$  deficiency and a variable reduction in  $\alpha$ -dystroglycan expression were observed.

## RESULTS

### Mutation screening of the *FKRP* gene in LGMD2I

Sequence analysis of the *FKRP* coding region identified mutations in individuals from 17 families, whose clinical features are summarized in Table 1 and mutations in Table 2. Unexpectedly, 15 families had an identical C826A (Leu276Ileu) mutation, of which five were homozygous for the mutation and the remainder were compound heterozygotes. None of the homozygous individuals was the offspring of consanguineous marriages. In the 10 families heterozygous for the C826A mutation we were able to identify the second allelic mutation in four cases. These were two missense mutations in families 7 and 9 (C934T and C947G), one nonsense mutation due to a 4 nt duplication in family 2 (390insTACC) and a 12 nt in-frame deletion (426del12) in family 10. In two families without the C826A mutation, we identified a homozygous G1016A missense mutation in family 1, a consanguineous family, and a C427A heterozygous missense mutation in family 11. Since the C826A mutation was present in a large number of families, we took advantage of the loss of a *BfaI* restriction site induced by the change (Materials and Methods; Fig. 1) to rule out this to be a polymorphism. On screening

controls, only one heterozygous individual was identified out of a total of 200 control chromosomes. We considered these mutations to be pathogenic since they were not present in at least 100 control chromosomes and segregated with the disease in an autosomal recessive fashion.

In eight cases we did not observe any changes in the *FKRP* coding sequence. Four of these individuals came from genetically informative families that were compatible with linkage to the *FKRP* locus. The other four were isolated cases.

### Haplotype analysis of families with the C826A mutation

We used markers *D19S219* and *D19S606* to haplotype families harbouring the C826A mutation, since radiation hybrid mapping suggested these were the closest markers flanking *FKRP* in the Genethon database. The order of markers is centromere-*D19S219*-*FKRP*-*D19S606*-telomere. The interval between the markers is 3 cM according to the Genethon map. Sequence data for this region of the genome puts *D19S606* as the closest marker, no more the 500 kb downstream of the *FKRP* gene. In 10 families haplotyping was possible to check for linkage disequilibrium with the C826A mutation. Amongst the families tested, there was not a conserved haplotype (Fig. 2). However, marker *D19S606*, the most closely linked marker to *FKRP*, identified two alleles at a higher than expected frequency. Allele 2 was found in five haplotypes and allele 5 in four haplotypes (compared to a frequency of 0.036 for allele 2 and 0.018 for allele 5 in the CEPH database).

### Immunohistochemistry and western blot analysis

The histological changes were characteristic of a muscular dystrophy in all patients. The expression of all proteins regularly screened for in the biopsies of patients with muscular dystrophy were normal. These included dystrophin,  $\alpha$ -,  $\beta$ -,  $\gamma$ -sarcoglycans, caveolin, dysferlin, emerin, lamin A/C and calpain 3 (data not shown). Immunocytochemical analysis of laminin  $\alpha 2$  using antibodies against both the 80 and 300 kDa fragments was reduced in several patients with a more severe phenotype (Fig. 3; Table 1), whereas it was normal in the remaining patients with the exception of few scattered fibres



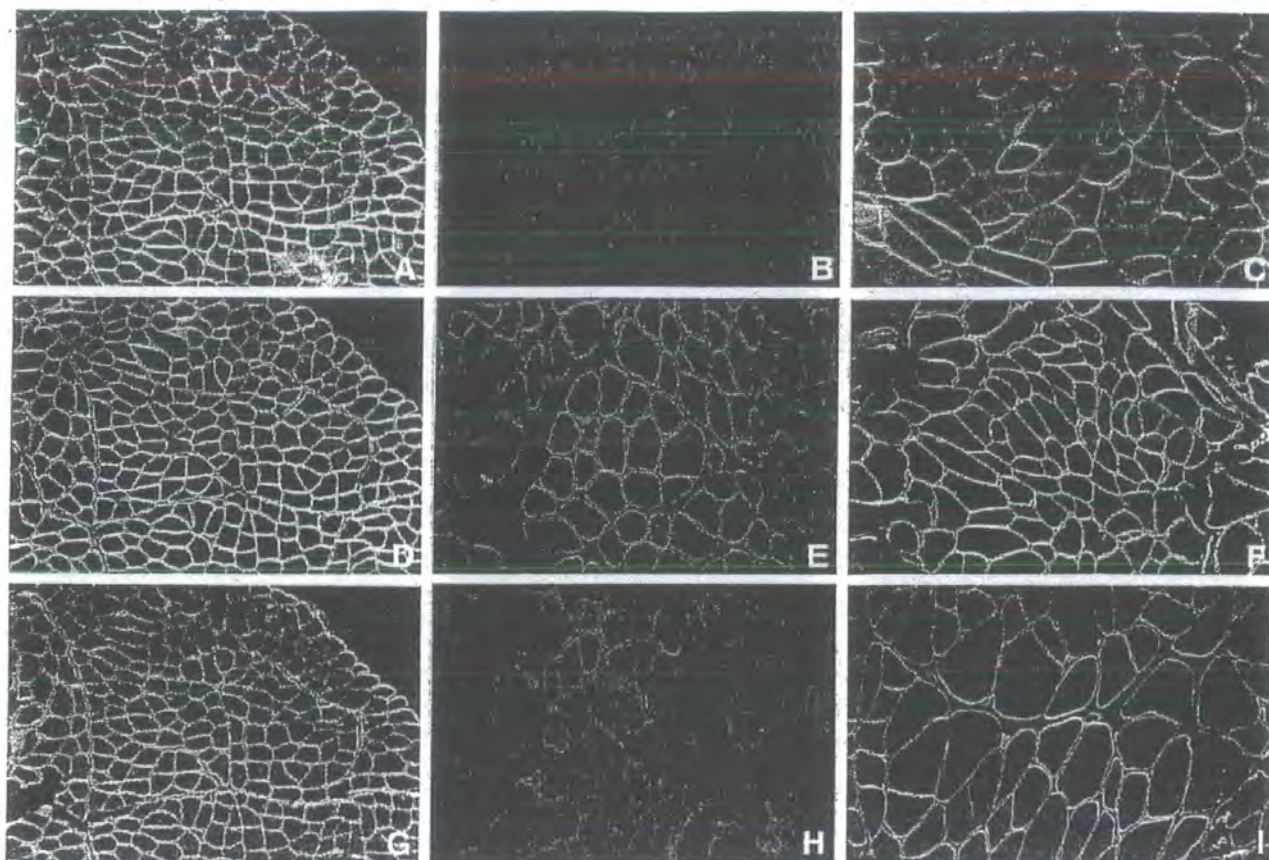


Figure 3. Immunocytochemical localization of  $\alpha$ -dystroglycan (A–C),  $\beta$ -dystroglycan (D–F) and laminin  $\alpha$ 2 (300 kDa) (G–I) in control muscle (A, D and G), family 5 (B, E and H) and family 15 (C, F and I).

(data not shown). Since  $\alpha$ -dystroglycan is a heavily glycosylated protein, we used two antibodies, V1A4-1 (Upstate Biotechnology) raised against an unknown epitope and a sheep polyclonal antibody, directed towards the core protein. Both antibodies clearly delineate individual muscle fibres with no apparent differences between fibre types in control muscle. In 10 patients with LGMD2I whose muscle was available to study,  $\alpha$ -dystroglycan expression was found to be abnormal (Table 1). This was more marked using the V1A4-1 antibody than with the antibody to the core protein in (Fig. 5A). The apparent reduction in  $\alpha$ -dystroglycan was more marked than laminin  $\alpha$ 2 (Fig. 3B). The pattern of  $\beta$ -dystroglycan labelling was normal, including those fibres in which  $\alpha$ -dystroglycan was reduced.

Western blot analysis using an antibody that recognized the 80 kDa merosin fragment revealed an absence or near-absence of labelling in family 15 (Fig. 4). This is identical to previous observations in this family, who along with families 13, 14 and 16, were reported as being affected by an 'adult merosinopathy' (18). The molecular mass of residual laminin  $\alpha$ 2 was normal, as was the expression of other proteins studied (Fig. 4). The virtual absence of laminin  $\alpha$ 2 on western blot was in marked contrast with the immunocytochemical analyses, where no reduction in its expression was evident.

## DISCUSSION

In this work mutations in the *FKRP* gene were identified in affected individuals from 17 families with LGMD2I. We originally demonstrated that mutations in this gene cause a severe form of CMD, MDC1C (15). The patients in this work had a variable phenotype, though all were less severe than MDC1C where patients never acquired independent ambulation. Patients at the severe end of the spectrum presented within the first 2 years of life with hypotonia, delayed motor milestones and followed by muscle hypertrophy (mostly in the legs, but also involving the tongue in several cases). These patients never acquired the ability to run or hop and followed a Duchenne-like disease course, with loss of independent ambulation in the early teens, followed by the development of cardiomyopathy. The remaining patients had milder phenotypes and are similar to those described in a large Tunisian family featured in the original description of LGMD2I (18). These patients were characterized by a later disease onset and a relatively benign course. Calf and to a lesser extent, thigh, brachioradialis and tongue muscle hypertrophy were present in most patients. Muscle cramps following exercise were also relatively common; one case had spontaneous myoglobinuria. Intelligence and brain MRI were normal. Serum CK was grossly elevated in all patients (between 10 and 30 times normal values) and the heart was variably affected, a feature not previ-



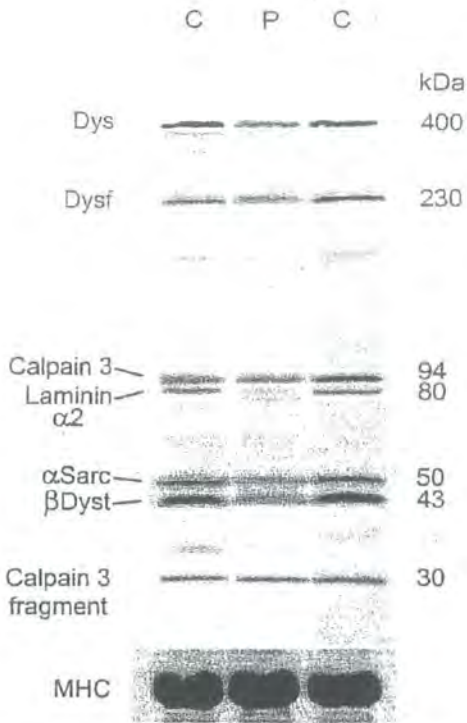


Figure 4. Multiplex western blot showing the marked reduction of the 80 kDa fragment of the laminin  $\alpha 2$  chain in family 15 (P) relative to controls (C). Protein loading of all samples was equivalent as shown by the staining of MHC on the gel after blotting.

ously reported in LGMD2I. The range of phenotypic severity due to *FKRP* mutations is surprisingly large and so far not observed in other forms of muscular dystrophy.

The *FKRP* mutations identified in the LGMD2I families were different from those seen in MDC1C. The most severe patient in this series was a consanguineous child from family 1 who was symptomatic in the first year of life and whose motor milestones were delayed. His phenotype was clearly intermediate between MDC1C and LGMD2I. Fifteen families had an identical C826A missense mutation. The five patients homozygous for this mutation had a relatively mild phenotype, whereas compound heterozygotes for this mutation on the whole had a more severe phenotype. Linkage analysis failed to identify a single founder haplotype associated with this mutation. However, we did observe two rare alleles of D19S606 that appeared to be in linkage disequilibrium with the mutation, suggesting it may have arisen on a limited number of occasions. Although we identified *FKRP* mutations in 17 families, we were unable to find mutations in four families that were consistent with linkage to this locus. In addition, we have failed to identify the second mutation in six of the 10 compound heterozygotes for the C826A mutation. This strongly suggests that mutations exist outside the *FKRP* coding region in at least some of these families and analysis of these regions is presently under way.

Patients at the severe end of the LGMD2I spectrum showed mild deficiency of laminin  $\alpha 2$  at the immunohistochemical level. Patients with a milder phenotype had normal expression of laminin  $\alpha 2$  on immunohistochemistry, but in four patients

western blot analysis identified a severe deficiency or total absence of this protein. These four families have been described previously by Bushby *et al.* (19). The discrepancy between the immunocytochemical and western blot analysis suggests that mutations in *FKRP* result in the altered processing, epitope masking or abnormal folding of the laminin  $\alpha 2$  polypeptide chain. The expression of  $\alpha$ -dystroglycan, but not  $\beta$ -dystroglycan, was abnormal in all muscle biopsies of LGMD2I patients available for study, an identical finding to MDC1C.  $\alpha$ - and  $\beta$ -dystroglycans are derived from post-translational cleavage of a single precursor peptide (20).  $\alpha$ -Dystroglycan is a heavily glycosylated peripheral membrane protein that forms a link between the actin associated cytoskeleton and, via laminin  $\alpha 2$ , the extracellular matrix (21,22). Laminin  $\alpha 2$  binds to a carbohydrate moiety of  $\alpha$ -dystroglycan (23). The pattern of  $\alpha$ -dystroglycan glycosylation differs not only between tissues but also within them, and different glycoforms may have different binding partners that mediate the function of  $\alpha$ -dystroglycan (23,24). Mutations in the *FKRP* gene appear to result in a selective deficiency of  $\alpha$ -dystroglycan in skeletal muscle, since the expression of other muscle proteins was normal. This is most likely due to an altered pattern of glycosylation, which would be expected to affect the function of  $\alpha$ -dystroglycan and be integral to the pathology in these patients. Recent evidence to support this comes from the abnormal expression of  $\alpha$ -dystroglycan in both FCMD (14) and the *myd* mouse, in which the LARGE glycosyltransferase gene is mutated (25). The deficiency of  $\alpha$ -dystroglycan represents an important biochemical marker in these conditions and in our experience is more sensitive than a deficiency of laminin  $\alpha 2$  expression.

Mutations in the *FKRP* gene account for a significant proportion of our patients with muscular dystrophy whose severity varied from a severe, congenital form characterized by inability to walk and early death (MDC1C), to a much milder form where patients are still ambulant in the fifth decade of life (LGMD2I). In our experience LGMD2I accounts for a large proportion of patients who have been previously excluded from other LGMD disease loci.

Most patients with a relatively mild disease course had a C826A mutation. As this mutation was mostly observed within two haplotypes, it is unlikely that it has arisen independently on multiple independent events. It may be there is some unknown selection pressure for the maintenance of this mutation within the population.

## MATERIALS AND METHODS

### Patients

Twenty-five families were studied after informed consent and approval of the local ethic committees. We have divided the case histories in two groups, depending on disease severity. Clinical information is summarized in Table 1.

### Patients with a severe phenotype (families 1–5)

In five families the clinical features were clearly more severe than in the remaining cases with a typical LGMD2I phenotype, with onset of symptoms before the age of 2 years. Despite the overlap with MDC1C, the disease course was significantly

milder when compared to this form of CMD, and resembled Duchenne muscular dystrophy in several aspects.

The clinical features in these five families were characterized by delay in early motor milestones, waddling gait, difficulties in running, mild facial weakness, calf and, in a few cases, tongue hypertrophy and Achilles tendon contractures. Intelligence and brain MRI were invariably normal whereas serum CK was markedly elevated (ranging from 1000 to 3160 IU/l, normal value < 190). Spontaneous bouts of myoglobinuria were observed in one case.

Three cases lost the ability to walk before the age of 14; evidence of cardiac involvement was present in four of these children. Additional features noticed in some families were soft and hyperextensible skin.

Families with a mild phenotype (6–17)

The remaining 12 families belonged to this group. Four of these families have been described in the past (19). There were a total of 16 affected individuals; all families were non-consanguineous. Early motor milestones were normal in each case. Onset of symptoms was in the first decade of life in one patient who complained of calf cramps aged 3 and enlargement of the calves. In the remaining patients onset of symptoms was in the second or third decade of life. Weakness affected the lower girdle causing waddling gait. With the exception of one patient, who deteriorated following an accident, making her wheelchair dependent, progression was not severe with all patients remaining ambulant. Mild facial and neck flexion weakness was present in most patients. Calf and, less commonly, brachioradialis hypertrophy were observed. Proximal muscles in the lower and upper limbs were weaker than distal muscles. There was a mild reduction in lateral flexion and rotation of the neck in several patients but otherwise no significant contractures. Serum CK was markedly elevated in all patients (1545–3696 IU/l, normal value < 140 IU/l). Cardiac involvement, in the form of reduction of left ventricular function, was noticed in three families. Cardiac involvement was also noticed in patients with MDC1C (15).

Linkage and haplotype analysis

DNA was extracted from whole blood using standard procedures after obtaining informed consent. Markers D19S606 and D19S219, separated by 3 cM on the Genethon map and spanning the FKRP locus, were used in initial linkage analysis. Primers amplifying these markers were purchased from Invitrogen, with the forward primer modified at the 5' end by the addition of either a FAM or NED fluorescent label. PCR products were separated on a 5% denaturing gel (Amresco) in an ABI 377A automated DNA sequencer (ABI) and analysed using Genescan v3.01 and Genotyper v2.01 software.

FKRP mutational analysis

A 1.7 kb fragment containing FKRP exon 4 and the entire coding sequence was amplified from genomic DNA using Advantage-GC Genomic Polymerase Mix (Clontech) and primers FKRP-1F (AAAGGGAATTGAGAAAGAGC) and FKRP-5 (GCTCACACAGAGCTTCTCC). PCR products

were separated by agarose gel electrophoresis, purified (Qiagen) and used for direct sequencing. Sequencing reactions were carried out using an ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and reverse primers FKRP-1R (GCAGGAAGGAGTCTACCAG), FKRP-2R (CCGAGAGGTTGAAGAGGT), FKRP-3R (CTCCTCGTAGAGGTAGGC), FKRP-4R (CCTTCTCCCATACGAAGC) and FKRP-5R.

The C826A mutation was further analysed by restriction enzyme analysis. A 900 bp DNA fragment was amplified using primers FKRP 2F (CCCAGAGGTTGAAGAGGT) and FKRP 4R and digested under the manufacturer's conditions with BfaI (New England Biolabs). PCR products were separated on 2% agarose gels. Wild-type DNA was cut into two fragments of 500 and 400 bp, whereas products harbouring the mutation, which introduced the loss of this BfaI site, remained uncut.

Immunocytochemistry

Unfixed frozen 8 µm sections were incubated with monoclonal antibodies to β-spectrin, laminin α2, α5, β1, γ1 (Chemicon), V1A4-1 (a monoclonal antibody raised against α-dystroglycan; Upstate Biotechnology), sheep polyclonal raised against α-dystroglycan. All primary antibodies were applied for 1 h and revealed with an appropriate biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min, followed by streptavidin conjugated to Alexa 594 (Jackson ImmunoResearch Laboratories) for 15 min. All dilutions and washings were made in phosphate buffered saline. Sections were mounted in aqueous mountant and viewed with epifluorescence using a Leica Aristoplan microscope. Control sections were labelled without primary antibodies, and all sections were compared with control samples from other neuromuscular disorders, and with normal muscle.

Western blotting

Muscle proteins were extracted in treatment buffer containing 0.125 mol/l Tris-HCL buffer pH 6.4, 10% glycerol, 4% SDS, 4 mol/l urea, 10% mercaptoethanol and 0.001% bromophenol blue (final pH of the treatment buffer was 6.8). Soluble proteins were separated using the Multiplex System of western blotting and then electrophoretically transferred onto nitrocellulose for 7 h as described previously (18). After blotting, the gels were fixed in 20% trichloroacetic acid and stained in 0.115% Coomassie brilliant blue R250 in 25% ethanol/10% acetic acid and destained in 10% ethanol/10% acetic acid. The density of the myosin heavy-chain band on the dried, post-blotted gel was used to indicate how much muscle protein had been loaded. For visualization of blotted proteins nitrocellulose strips were blocked in 5% milk powder in a pH 8 buffer containing 10 mmol/l Tris-HCL, 0.15mol/l NaCl and 0.05% Tween 20 (TBST). Blots were probed with antibodies to dystrophin [Dy8/6C5, calpain 3, α-sarcoglycan, β-dystroglycan (all available from Novocastra Laboratories) and laminin α2 chain (80 kDa fragment; Chemicon)] and visualized using peroxidase-conjugated anti-mouse secondary antibody followed by exposure to freshly prepared 0.05% diaminobenzidine.

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